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Endothelial Heterogeneity Associated with Regional Athero-Susceptibility and Adaptation to Disturbed Blood Flow in Vivo

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Abstract

Endothelial phenotypic heterogeneity plays an important role in the susceptibility of the cardiovascular system to disease. Arteries and heart valves are susceptible to chronic inflammatory disease in regions of blood flow disturbance that implicates hemodynamic forces and transport characteristics as prominent influences on endothelial phenotype. By combining in vivo high-throughput genomics (discovery science) and in vitro mechanistic approaches (reductionist science), we present endothelial patho-susceptibility as an imbalance of multiple interrelated pathways that sensitize the cells to pathological change. The recently identified association of endoplasmic reticulum stress with endothelium in regions of flow disturbance is outlined as an important example of susceptible phenotype linked to proinflammatory and oxidative stress pathways.

Keywords

Endothelium; hemodynamics; athero-susceptibility; gene expression; shear stress

“The application of omics to common cardiovascular diseases has potential obstacles. The effects on biomarker concentrations are likely to be far more subtle than in the case of congenital errors of metabolism, and the extent of individual variability ... remains unclear.”

Gerszten and Wang¹

Atherosclerosis is a disease of diverse pathologies and complex etiology that traditionally has been addressed by histological classification of lesions² and by biochemical markers of inflammation, immune reaction, and lipid metabolism.^{3,4} Knockout and transgenic animals provide valuable information about the complex consequences of the effects of (usually) a single gene manipulation; polymorphisms linked to cardiovascular disorders provide valuable associations with subtle clinical effects.⁵ However, pathological change at the

vascular cellular level, particularly the endothelial interface, is lumped into criteria of dysfunction that tend to be centered around the expression of a primary molecule or pathway, notably those associated with vasoactivity, inflammation, or lipid metabolism. As inferred by Garszten and Wang,¹ quoted above, it must be recognized that an extensive range of possible metabolic states of the endothelium, some more pathologically relevant than others, are associated with the susceptibility to, and the initiation and development of, atherosclerosis and that these are a regional (and perhaps focal) manifestation of phenotypic heterogeneity in vivo. Definition of disease susceptibility therefore is strongly influenced by the local in vivo environment; for the endothelium this includes important contributions by the spatial and temporal characteristics of blood flow.

An important determinant of athero-susceptibility and its corollary, athero-protection, is the local geometry of arteries that in turn regulates the hemodynamic force distribution and cell surface transport characteristics to which the endothelium is highly sensitive,^{6,7} a prominent environmental influence. In regions of unstable flow separation associated with curvatures (Fig. 1A and 1B), branches (Fig. 1C), and bifurcations in the arterial circulation as well as the downstream side of heart valves (Fig. 1D) where the average shear stress is low, steep temporal and spatial gradients of shear stress are associated with oscillatory flow reversal. Because multidirectional forces act on the cells at these sites of disturbed flow, the endothelium, unlike elsewhere in the arterial circulation, lacks preferential cell alignment and often expresses a polygonal morphology. Disturbed flow regions correlate closely with susceptibility to pathological change: atherosclerosis in arteries and calcific sclerosis in heart valves. Endothelium at such locations expresses significant differences in gene expression compared with adjacent undisturbed laminar flow regions.^{8,9} These differences are proposed to be an important influence on susceptibility to, or protection from, pathological change^{10,11} mediated by the endothelium.

Flow-phenotype relationships were initially addressed by gene expression studies using cultured endothelium.^{12–14} Candidate genes identified in vitro have been demonstrated to be important in vivo.^{15–19} However, high-throughput methodologies applied to small regions of the endothelium⁸ can define endothelial phenotypes and can more fully address the in vivo context.

Endothelial phenotypes in athero-susceptible sites can be considered to reflect the kinetic status of multiple interacting cellular pathways in cells that are adapted to the locally complex flow characteristics. Because the interpretation of gene expression networks in vivo is informative for optimal design of in vitro experiments that address the underlying mechanisms, a combined in vivo/in vitro approach greatly expands the relevance of in vitro investigations alone.

FUNCTION TO DYSFUNCTION

The subtle and complex pathways that determine functional phenotype in vivo are reflected in multiple levels of regulation of gene and protein expression. There is a broad range of endothelial phenotypes in arteries—and, pertinent to this discussion, within the same artery—each arising from the complex interplay of cellular pathways that regulate function. Unbiased genomic analyses have shown that athero-susceptible and athero-protective regions express significant differences of endothelial phenotypes before any pathology develops.^{8,20–26} The transition from normal phenotype to overt dysfunction at these sites is not defined; it is generally assumed to be a threshold event but may be a continuum of functional variation.

An understanding of the mechanisms that determine the transition of endothelial function to dysfunction ideally requires (1) an appreciation of the heterogeneity of normal endothelial

cells at lesion-susceptible sites before any pathology develops, (2) an estimation of the normal steady-state transcriptome and proteome of the cells, and (3) measurement of the dysequilibrium that may occur when pro-pathological challenges are introduced.

IS THE ENDOTHELIUM SENSITIZED FOR ATHEROGENESIS AT ATHERO-SUSCEPTIBLE SITES?

Both global and targeted analyses of endothelial gene and protein expression in swine^{8,20–26} and mice^{9,27} have shown significant differential regional expression at sites where the initiation and development of atherosclerosis is predictable, when compared with locations that are resistant to atherogenesis. The coexistence of pro- and anti-atherosclerotic gene expression in endothelium of athero-susceptible sites in swine⁸ and the increased expression of proinflammatory proteins in mice⁹ in the absence of obvious vascular inflammation suggested that the endothelium at athero-susceptible sites is sensitized, or “primed,”⁹ to develop inflammation but that the presence of additional risk factors is required to initiate disease.

Like most cells, endothelium is tolerant of adverse environmental conditions. Regions of athero-susceptibility are associated with the complex hemodynamic force characteristics and solute transport created by the local vascular geometry. Our working model is that the cells adapt to these conditions while retaining key characteristic endothelial functions; however, they are in a different phenotypic state that confers susceptibility to pathological change. Spatially sensitive differential expression of intracellular pathways may represent a chronic adapted state; in other words, the dynamic states of many individual steps in interconnected pathways are regionally different and the cells are in a different equilibrium state overall but still within a tolerable range of normal function.

In regions of athero-susceptibility, disturbed flow is itself an endogenous chronic risk factor; however, it is insufficient to lead to atherogenesis in the absence of additional systemic risk factors. Athero-pathology occurs as the result of progressive imbalance until (1) the consequences of chronic disequilibria create functional imbalances that promote pathogenesis, and/or (2) an intracellular threshold is reached that activates an endothelial-specific inflammatory response. The equilibrium model predicts that the cellular balance in these locations will be tipped to pathological states when challenged by hypercholesterolemia, diabetes, homocysteinemia, smoking, hypertension, or other systemic risk factors.

Here is a working definition of sensitized endothelial phenotype: The endothelial cell is an integrated system of dynamic biophysical and biochemical events under regulatory control. However, considerable latitude is afforded by protective regulatory checks and balances, and a wide range of metabolic conditions is tolerated within normal endothelial function. In sensitized endothelium, the dynamic balance is shifted toward the extremes of the range, which for this discussion is toward an inflammatory phenotype.

Illustrations of this concept are in vivo profiling of inflammatory networks and the recent in vivo and in vitro evidence for activated endoplasmic reticulum (ER) stress/unfolded protein response (UPR) identified in endothelium of athero-susceptible regions,^{24,28,29} which appears to be a chronic adaptive (pre-pathological) response to local flow disturbance and is linked to oxidative stress, inflammation, and apoptosis (discussed later).

REGIONAL TRANSCRIPTOME PROFILES IN VIVO

In early studies,⁸ cells from the athero-susceptible inner curvature of the swine aortic arch (AA), where flow separations lead to oscillatory flow reversal and low average shear stress (collectively disturbed flow), were compared with nearby endothelium of the descending thoracic (DT) aorta, a region where the flow was unidirectional and the average shear stress was higher (undisturbed flow). Following endothelial cell isolation, RNA was isolated, purified, and linearly amplified and then labeled for hybridization to cDNA- or oligo-microarrays and analyzed. Significant differences were identified in the phenotypes of endothelium located in susceptible AA regions when compared with cells at protected sites.⁸ Surprisingly, the coexistence of enhanced expression of pro-pathological inflammatory genes and protective antioxidant genes was noted in the same cells in athero-susceptible regions, reflecting a balanced phenotype consistent with normal function but one that is set at a different equilibrium state than in regions protected against atherogenesis. Multiple genes associated with the nuclear factor kappa binding (NF- κ B) protein pathway were expressed in a proinflammatory direction despite the absence of pathway activation. In the same cells, many genes were expressed that promote an antioxidative profile inhibitory of NF- κ B activation. In a similar response, several differentially expressed procoagulation genes were balanced by expression of anticoagulation molecules.

ENDOTHELIAL ENDOPLASMIC RETICULUM STRESS IN ATHERO-SUSCEPTIBLE LOCATIONS

In an extension of the AA/DT comparison,²⁴ we investigated whether athero-susceptibility can be associated with phenotype characteristics common to a variety of arteries. In a multisite study in 45 normal adult swine, susceptible regions of AA, proximal brachiocephalic artery, aorto-renal branch region, and abdominal aorta were analyzed relative to protected sites of the common carotid artery, DT, and the distal renal artery. All athero-susceptible regions are associated with complex disturbed blood flow. From this multisite study the most abundant common feature of the endothelium of all athero-susceptible regions was the upregulation of genes associated with ER processing of proteins, ER stress, and the UPR. Differential gene expression analysis identified 133 genes, 73% of which are involved in ER protein processing and folding and form a highly connected and coordinated network of genes upregulated in the susceptible regions (Fig. 2). Three independent and unbiased pathway mining approaches—Gene Ontology using the program DAVID (National Institutes of Health, Bethesda, MD), Gene Set Enrichment Analysis (GSEA), and Ingenuity Pathway Analysis (Ingenuity Systems, Inc., Redwood City, CA)—identified ER stress and the UPR to be overrepresented functional categories in athero-susceptible endothelium including genes that function in protein folding, synthesis, and posttranslational protein modification as well as inflammation and apoptosis linked to ER stress.³⁰ ER stress results in the activation of the UPR, an ubiquitous adaptive cell response that assists cell survival in an adverse environment by activating a set of intracellular signaling pathways in response to cellular stress.³¹ This arises because of excessive protein biosynthesis or interference with normal protein folding mechanisms in the ER lumen. Excessive newly synthesized and/or mis-folded polypeptides in the ER lumen exceed its protein folding capacity. The UPR signals a coordinated transcriptional upregulation of ER chaperones and folding enzymes to promote the correct assembly of unfolded polypeptides and prevent incompletely folded proteins from aggregating (Fig. 3).

In the unstressed state, the ER chaperone heat-shock protein A5, HSPA5 (also known as binding protein, BiP, and glucose-related protein 78, GRP78) binds to each of three ER stress transducers.³² These are ER transmembrane proteins each having an ER-luminal domain for the sensing of unfolded proteins and a cytosolic domain for signaling. While

bound, HSPA5 maintains the inactive state of the transducers; however, when an imbalance occurs in the luminal flux of newly synthesized unfolded or misfolded peptides as a result of cell stress, the UPR is activated. HSPA5 dissociates from the chaperones and binds unfolded/misfolded polypeptides in the ER lumen releasing inhibition of the transducers. Activation of ATF6 (activating transcription factor 6), IRE1 (inositol requiring kinase 1), and PERK (protein kinase-like ER kinase) and the downstream consequences of activity constitutes the UPR as outlined in Fig. 4A. Activated ATF6 is transported to the Golgi apparatus where it is cleaved by proteolysis to a 50-kDa fragment that moves to the nucleus to activate transcription of UPR target genes. IRE1 dimerizes and self-phosphorylates, gaining endoribonuclease activity that splices XBP-1 (X-Box-binding protein-1) mRNA. Spliced XBP-1 encodes the XBP-1 transcription factor, which translocates to the nucleus to activate target genes. Dimerization and phosphorylation also activates PERK that phosphorylates eIF2 (eukaryotic initiation factor 2), which results in translational attenuation of most proteins with the exception of ATF4 (activating transcription factor 4). ATF4 translocates to the nucleus where it binds the UPR response element. The products of these activated UPR transducers converge as transcriptional regulators in the nucleus to upregulate ER chaperones and UPR transducer synthesis and to ubiquitinate unfolded proteins for degradation through the proteasome; 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 the transcription factor CHOP, inflammation through activation of NF- κ B, and generation of reactive oxygen species (ROS) through excessive protein oxidation in the ER.³²

In the multisite endothelial transcriptome study, many genes associated with protein biosynthesis, ER stress, and the UPR were selectively enhanced in regions of athero-susceptibility;²⁴ the transducer biochemistry was therefore investigated further. To validate the global genomics analyses, endothelial cell proteins were isolated from AA and DT and also from the athero-susceptible aorto-renal branch and the protected distal renal artery. At each athero-susceptible disturbed flow site, HSPA5 transcript and/or protein expression was significantly upregulated (Fig. 4B). Expression of activated proteins in two of the three transducer pathways measured by Western blot demonstrated significantly elevated phospho ATF6, phospho IRE1 and its target, spliced XBP-1. However, the third transducer pathway PERK was not activated; the expression of phosphorylated PERK and its downstream effectors phospho eIF2 and ATF4 were similar in endothelium from all locations.²⁴ Activation of both ATF6 and PERK induces the pro-apoptosis transcription factor CHOP (C/ERB homologous protein) which was increased two- to threefold; however, it appears that the upregulation of CHOP measured in athero-susceptible sites is attributable to ATF6 and not to PERK. In support of this conclusion, PERK phosphorylation of Nrf2 (nuclear factor erythroid 2-like 2), a transcription factor that upregulates antioxidative genes,³³ was similar at susceptible and protected sites. Overall, this study, approached without preconceived expectations of differential expression of genes and proteins associated with ER stress/UPR, strongly suggests that stresses associated with flow disturbance in vivo elicit partial activation of the UPR, an ER response common to other forms of stress, and that chronic stress is a signature for athero-susceptible endothelial phenotype.

Two other reports support these conclusions. Feaver et al²⁸ demonstrated increased expression of the ER-stress chaperone binding protein GRP78 (HSPA5; BiP) in AA, branch sites, and lesions of apoE^{-/-} mice. Recently, Zeng et al,²⁹ focusing on the XBP-1 branch of UPR, demonstrated endothelial expression in branching regions of apoE^{-/-} mice arteries. In both studies, there is substantial ER-stress-related protein expression in other cells of the arterial wall in athero-susceptible sites. Overexpression of spliced (activated) XBP-1

(sXBP-1) induced apoptosis in cultured human endothelial cells and, following adenoviral-mediated overexpression of endothelial sXBP-1 in an apoE^{-/-} murine aortic iso-graft model, atherosclerosis developed in normally protected regions of the vessel.²⁹ Collectively, these three complementary studies support a role for endothelial ER stress in athero-susceptibility and lesion development. A causal relationship between flow characteristics and the stress responses has recently been addressed.

FLOW DISTURBANCE INDUCES ENDOPLASMIC RETICULUM STRESS AND UNFOLDED PROTEIN RESPONSE

The application (to human umbilical vein endothelial cell) of an athero-susceptible flow waveform based on that measured in human carotid bifurcation resulted in a sustained upregulation of GRP78/HSPA5 together with an increase in protein stability.²⁸ Some evidence for ATF6 induction and for activation of the ER stress sensing element were also reported in this study. In XBP1-centric experiments,²⁹ an approximation of disturbed flow was used to expose cultured endothelial cells to spatial and temporal gradients to simulate flow disturbance. Increased expression of both spliced and unspliced forms of XBP1 were reported in disturbed flow. This may be linked to endothelial proliferation because knockdown of XBP1 inhibited Br-dU incorporation. Endothelium in athero-susceptible sites express a small but significant increase in proliferation³⁴ consistent with enhanced permeability, the implication being that ER stress may contribute to permeability via focally enhanced cell turnover in these regions.³⁵ It appears reasonable to conclude that flow contributes to an altered regional phenotype of ER stress/UPR in athero-susceptible endothelium, but the detailed relationships and regulatory mechanotransduction mechanisms remain poorly understood.

ENDOPLASMIC RETICULUM STRESS AND OXIDATIVE MECHANISMS

There is an association between ER stress and ROS, but it is probable that ER stress both produces ROS and that ROS in turn induce ER stress.³⁶ Oxidative stress, the generation of highly reactive ROS molecules, occurs during protein folding through the formation of intra-molecular and intermolecular disulfide bonds;³⁷ molecular oxygen is the terminal electron recipient during disulfide bond formation. ROS scavenger molecules such as reduced glutathione that neutralize ROS are depleted in the UPR, further adding oxidative stress.³⁸ Conversely, ROS are themselves highly reactive stress-inducing agents that, when locally generated through other mechanisms, add to the ER-stress burden.³⁶ For example, hydrogen peroxide, the end product of multiple protective responses, is a potent ER-stress inducer, and oxidized phospholipids induce ATF4 protein levels, XBP1 splicing, and ATF6 cleavage in cultured human arterial cells.³⁹ In endothelium, GRP78/HSPA5 sensitivity to peroxynitrite and its colocalization with 3-nitrotyrosine in atherosclerotic lesions of apo E^{-/-} mice has been demonstrated.³⁹ *En face* confocal imaging of swine artery endothelium *ex vivo* to measure ROS accumulation as nuclear accumulation of dihydroethidium demonstrated that many more cells (16-fold) in the athero-susceptible AA accumulate ROS than cells in a nearby (protected) descending thoracic aorta (Fig. 5). The finding is consistent with upregulation of UPR. An enhanced oxidative load may activate inflammation in these regions.

ENDOPLASMIC RETICULUM STRESS AND INFLAMMATION THROUGH NFκB ACTIVATION

In addition to ROS, events in the ER are linked to inflammation through various other mechanisms. They include ER calcium regulation, activation of the NF-κB pathway, mitogen-activated protein kinases, and acute immunological reactions.³⁸ In the context of

endothelial athero-susceptibility, components of the NF- κ B pathway have been implicated in athero-susceptibility for more than a decade, initially through the activation of NF- κ B and AP-1 (transcription factor activator protein 1) by flow in vitro,^{40,41} subsequently by direct measurement of nuclear translocation of NF- κ B in mice AA^{9,42} and the strong association between NF- κ B pathway genes and athero-susceptible endothelium in swine aorta.^{8,43} NF- κ B is normally held inactive in the cytosol as a complex with I- κ B, a family of inhibitors of NF- κ B. Upon phosphorylation, I- κ B is degraded, releasing NF- κ B for translocation to the nucleus where it regulates proinflammatory genes. An increased ER processing load and oxidative stress have been proposed as a link between ER stress and NF- κ B activation. The UPR transduction pathway through PERK-EIF2 α inhibits translation and has been proposed to favor activation of NF- κ B by causing an increase in the NF- κ B-to-I- κ B ratio because of the longer half-life of NF- κ B compared with I- κ B.⁴⁴ However, a more direct mechanism linking UPR to NF- κ B may occur through IRE1 α , which as noted earlier is one of the two chronically activated transducer pathways in swine athero-susceptible endothelium.

When IRE1 α is activated by autophosphorylation in the UPR response, a conformational shift allows it to bind TRAF2 (tumor necrosis factor [TNF] receptor-associated factor 2).⁴⁵ The IRE1 α -TRAF2 complex binds IKK (I- κ B kinase), which phosphorylates I- κ B leading to its degradation, releasing NF- κ B for translocation to the nucleus. The IRE1 α -TRAF2 complex also binds the kinase JNK, which in turn phosphorylates transcription factor AP1. In the nucleus, both NF- κ B and AP1 induce transcription of inflammatory genes. In a recent study⁴⁶ we investigated endothelial phenotype heterogeneity of microRNA (miR) expression. miRs are highly conserved, noncoding small RNAs of ~22 nucleotides that direct posttranscriptional repression. Mammalian miRs pair to the 3' untranslated region of target mRNAs, promoting transcript degradation and/or inhibiting protein translation of the protein-coding genes. The miRs 10a and 10b were measured to be significantly suppressed in the inner AA endothelium of normal swine (20% of the expression levels measured in the descending thoracic aorta). Associations between miR10a expression and regulatory enzymes for I- κ B were established through *in silico*, *in vivo*, and *in vitro* experiments (Fang Y et al; manuscript submitted), suggesting that upstream regulation of some elements of the ER-stress-ROS-NF- κ B-inflammation network may occur through small inhibitory RNAs that are differentially expressed in athero-susceptible locations.

PROTECTIVE RESPONSES OF THE ENDOTHELIUM TO EARLY SYSTEMIC RISK FACTOR EXPOSURE (HYPERCHOLESTEROLEMIA)

Because the ER-stress response is partially activated in endothelium at athero-susceptible sites, it might be expected that further disequilibria induced by atherosclerosis risk factors such as hypercholesterolemia when added to the biomechanical stress of disturbed blood flow will push the dynamic balance to a proinflammatory state (JNK/NF- κ B activation) or to apoptosis. However, when adult swine were fed a hypercholesterolemic (HC) diet for a brief period (2 weeks), ER stress within the endothelium of athero-susceptible arteries was not greater than that of the corresponding susceptible sites in normocholesterolemic swine.²⁶ In the same study, it should be noted that the ER-stress marker differences between susceptible and protected sites persisted in both diets (Fig. 6). The cholesterol-exporting protein ATP-binding cassette A1 (ABCA1) was universally upregulated to deal with the increased cholesterol load irrespective of susceptible or protected location, indicating that the arterial endothelium responded to the HC diet, but the adaptive changes to HC appeared to protect against pro-pathological gene expression, and very few other genes were differentially expressed.

In the aortic valve endothelium, a similar, but more marked, protective adaptation to that identified in arteries occurred in short-term hypercholesterolemia. Here, the side of the valve

adjacent to the ascending aorta is highly susceptible to inflammatory aortic valve disease, whereas the other (ventricular) side is resistant to disease. We have demonstrated that the endothelium of the normal aortic side is phenotypically distinct from that of the ventricular side, expressing a balance of pro- and anti-inflammatory transcripts and a pro-calcific profile.²¹ When challenged by short-duration HC, localized lipid insudation was confined to the aortic side of the leaflet. Transcript profiling of valve endothelial populations demonstrated that the susceptible aortic side was much more sensitive to 2 weeks of HC than the ventricular side; 1325 versus 87 genes were differentially expressed on the aortic and ventricular sides, respectively, by HC when each side was referenced to normocholesterolemia.²⁵ Side-specific sensitivity to HC, however, was not evidence of dysfunctional phenotype. Instead, pathway analyses identified differential expression of caspase3-, peroxisome proliferator-activated receptor α -, TNF α -, and NF κ B-related pathways that were consistent with a protective endothelial phenotype, a finding confirmed at the protein level.²⁵

The studies just cited were short-term exposure to HC. Susceptible regions of the arteries and valve eventually develop a well-documented inflammatory response when sustained HC leads to the development of atherosclerosis or aortic valve sclerosis. Ongoing studies that extend the duration of HC exposure will determine at what stage inflammation replaces the adaptive protective mechanisms and whether this is an abrupt transition or gradual rebalancing of the endothelial phenotype.

CONCLUSION

In summary, the endothelial phenotypes in undiseased but patho-susceptible regions of arteries and heart valves share some fundamental characteristics that distinguish them from patho-protected sites. The presence of disturbed blood flow may sensitize or prime these regions through biomechanical forces and transport imbalances (e.g., the retention of locally generated ROS). Whatever the primary mechanism(s), the endothelium in these regions in arteries is experiencing a local environment that increases the protein biosynthetic load and promotes ER stress and the UPR. These are linked to classic pathways of ROS, inflammation, cell proliferation, and apoptosis that are not fully activated unless additional risk factors are introduced over a sustained period. Preliminary evidence suggests that in the case of short-duration hypercholesterolemia, paradoxical protective measures are induced in response to further stress.

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Figure 1. Swine arterial and aortic valve sites of disturbed flow/athero-susceptibility (rotating arrows) and unidirectional shear stress/athero-protection (straight arrows). (A) Anatomy of a portion of the mammalian arterial circulation. (B) Inner curvature of the athero-susceptible aortic arch and protected descending thoracic aorta in (B1) unopened and (B2) opened view. (C) Athero-susceptible aorto-renal branch region and protected distal renal artery in (C1) unopened and (C2) open view. (D) Aortic valve leaflet and outline of undisturbed and disturbed flow. Schematic: In athero-susceptible sites, a range of endothelial phenotypes, influenced in part by local flow characteristics, exist in balance between protected and pathological states.

Figure 2.

Ingenuity Pathway Analysis (Ingenuity Systems, Inc., Redwood City, CA) of endothelial gene expression. Following microarray analyses, a data set containing gene identifiers with corresponding expression values and False Discovery Rates were analyzed using Ingenuity Pathway Analysis. Networks of these focus genes were then algorithmically generated based on their connectivity. Only direct interactions of gene–protein and protein–protein were considered. Seven networks were combined to form one large network. Dark gray color indicates EC gene upregulation in athero-susceptible regions of arteries compared with athero-protected regions. Seventy percent of these genes were interrelated through protein biosynthetic pathways. Light gray color indicates molecules present in the data set that were not significantly differentially expressed.

Figure 3.
Outline of endoplasmic reticulum (ER) stress mechanism.

Figure 4.

Endoplasmic reticulum (ER) stress and unfolded protein response (UPR). (A) UPR signaling outline. (B) ER stress marker HSPA5 (BiP) gene and protein (78 kDa) expression in aortic arch (AA), normalized to descending thoracic (DT) aorta, for each paired sample based on their animal origin. Gene ($n = 6$ paired samples) and protein ($n = 12$ paired samples) expression was normalized to GAPDH and β -actin, respectively. Values >1.0 indicate higher expression in AA. Data represent mean $\pm$ standard error of mean. * $p < 0.05$ one-sample, one-sided, paired Wilcoxon test. (Reprinted with permission from Civelek M, Manduchi E, Riley R, Stoeckert CJ, Davies PF. Chronic endoplasmic reticulum (ER)-stress activates unfolded protein response (UPR) in arterial endothelium in regions of susceptibility to atherosclerosis. *Circ Res* 2009;105:453–461.)

Figure 5.

Reactive oxygen species (ROS) detection in aorta. (A) Freshly harvested swine arteries were incubated with dihydroethidium (nuclear stain for ROS) and isolectin B₄ (outlines cell junctions). The endothelium of the athero-susceptible inner curvature of the aortic arch (AA) and athero-protected descending thoracic (DT) aorta were imaged *en face* using confocal microscopy under identical conditions. (B) Percentages of endothelial cells expressing nuclear ROS staining within the AA and DT plotted as a ratio.

Figure 6.

Endoplasmic reticulum (ER) stress and unfolded protein response (UPR) pathway gene expression in normal and hypercholesterolemic animals. Transcript expression (quantitative real-time polymerase chain reaction) was normalized to GAPDH in normal and hypercholesterolemic animals in two different regions. NC, normocholesterolemia; HC, hypercholesterolemia. Data are presented as mean $\pm$ standard error of mean. Significance was assessed by Student *t* test. No significance was found between NC and HC samples. (Reprinted with permission from Civelek M, Grant GR, Irolla CR, et al. Prelesional arterial endothelial phenotypes in hypercholesterolemia: universal ABCA1 upregulation contrasts with region-specific gene expression in vivo. *Am J Physiol Heart Circ Physiol* 2010;298(1):H163–170.)