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# Chronic endoplasmic reticulum stress activates unfolded protein response in arterial endothelium in regions of susceptibility to atherosclerosis

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## Abstract

**Rationale—**Endothelial function and dysfunction are central to the focal origin and regional development of atherosclerosis; however, an *in vivo* endothelial phenotypic footprint of susceptibility to atherosclerosis preceding pathological change remains elusive.

**Objective—**To conduct a comparative multi-site genomics study of arterial endothelial phenotype in athero-susceptible and athero-protected regions.

**Methods and Results—**Transcript profiles of freshly isolated endothelial cells from 7 discrete arterial regions in normal swine were analyzed to determine the steady state *in vivo* endothelial phenotypes in regions of varying susceptibilities to atherosclerosis. The most abundant common feature of the endothelium of all athero-susceptible regions was the upregulation of genes associated with endoplasmic reticulum (ER) stress. The unfolded protein response (UPR) pathway, induced by ER stress, was therefore investigated in detail in endothelium of the athero-susceptible aortic arch and was found to be partially activated. ER transmembrane signal transducers IRE1α and ATF6α and their downstream effectors, but not PERK, were activated concomitant with a higher transcript expression of protein folding enzymes and chaperones, indicative of ER stress *in vivo*.

**Conclusions—**The findings demonstrate the prevalence of chronic endothelial ER stress and activated UPR *in vivo* at athero-susceptible arterial sites. We propose that chronic localized biological stress is linked to spatial susceptibility of the endothelium to the initiation of atherosclerosis.

### Keywords

hemodynamics; DNA microarrays; gene expression

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## INTRODUCTION

Atherosclerosis originates as focal arterial lesions having a predictable distribution to regions of bifurcations, branches and inner curvatures [1]. Here, blood flow separates from unidirectional laminar flow to create complex laminar and occasional turbulent flow collectively referred to as disturbed flow [2, 3]. Functional and structural endothelial responses to systemic atherosclerotic risk factors within large arteries alter endothelial permeability, coagulation balance, and vasoactive properties of the arterial wall[4]. However, the initiation of atherogenesis is localized to specific susceptible sites. Endothelial cells display phenotype heterogeneity reflecting the various functions they perform in different regions of the circulation[5] and they play a key role in the initiation and progression of lesions[3] but the mechanisms that link regional heterogeneity and atherosusceptibility *in vivo* are poorly understood. Previous *in vitro* 6 and *in vivo* 7 studies have established a link between complex hemodynamics and athero-susceptible endothelial phenotypes. However, a molecular signature for spatial susceptibility *in vivo* has not yet been established, principally because few *in vivo* phenotyping studies have been performed.

The effects of the local hemodynamic environment on EC phenotypic heterogeneity have been implicated through multiple *in vitro* studies that provide extensive information on flow-induced endothelial responses [6, 8–10]. However, most *in vitro* flow devices designed to recapitulate *in vivo* flow conditions fail to simulate one or more components of the complex arterial flow fields [11]. An *in vivo* EC profiling study showed complex gene expression with the coexistence of both pro- and anti-atherosclerotic pathways in the athero-susceptible aortic arch region of normal swine [7], and the dominance of site specificity over gender or diet as a determinant of phenotype was demonstrated in a follow-up study [12]. Overall, these studies suggest that the presence of disturbed flow is associated with a steady-state EC phenotype that is primed for the initiation of atherosclerosis, but a common mechanism of athero-susceptibility at multiple sites *in vivo* has not been identified.

Here, we profile endothelial phenotypes of normal adult swine arteries at multiple regions (Fig. 1) that are susceptible or resistant to atherosclerosis in similar locations to those of humans [13]. Each susceptible site is associated with complex disturbed flow characteristics. Using unbiased genomic analyses we identify ER-stress-related protein biosynthesis as the prevalent endothelial genomic signature in all athero-susceptible locations. Biochemical measurements in endothelium of the athero-susceptible aortic arch demonstrated activation of two of the three UPR signaling pathways. Together the studies demonstrate that chronic ER stress characterizes the pre-pathological state of athero-susceptible endothelial phenotypes *in vivo*.

## METHODS

The complete MIAME compliant annotated study has been deposited into the public repository ArrayExpress (accession number E-CBIL-42). Detailed MIAME compliant annotation and data for this study are also available for user-friendly querying at www.cbil.upenn.edu/RAD (RAD study_id=3265). Supplementary information and files are available online.

### Sample collection

Fifty five endothelial cell (EC) samples, 7–8 from each of 7 distinct arterial regions (Fig. 1a,b), were collected by gentle scraping from 45 different adult swine shortly after sacrifice at a local abattoir (6 months-old, ~250 lbs, Hatfield Industries, Hatfield, PA). EC purity was 96.5% with 2.8% smooth muscle cell and 0.7% leukocyte contamination (Fig. S1). Regions showed normal histology without inflammation or lipid insudation. Cells were transferred to lysis buffer for RNA and protein extraction.

### Microarray hybridization

High quality total RNA was linearly amplified and hybridized to custom-printed porcine microarrays (ArrayExpress A-CBIL-16). Cy5-labelled sample mRNA was combined with Cy3-labelled common reference RNA that consisted of aRNA amplified from pooled total RNA from all regions. Microarrays were scanned with an Agilent DNA Microarray Scanner and the images were analyzed with Agilent Feature Extraction Software (version 9.1).

### Bioinformatics

Data pre-processing is described in the Supplementary Information. Differential expression analysis of genes was performed using Patterns of Gene Expression (PaGE v5.1.6) [14]. Genes with less than 25% false discovery rate (FDR) were considered to be differentially expressed. The list of differentially expressed genes was interrogated for statistically significant over-represented biological themes using DAVID [15] and for network formation and direct interactions using Ingenuity Pathway Analysis (IPA) [16]. Differential expression analysis of gene sets was performed using Gene Set Enrichment Analysis (GSEA)[17].

### Quantitative real time PCR and western blots

A separate cohort of 10 animals were used to isolate ECs from aortic arch (AA), thoracic aorta (DT), renal branch (RB) and renal artery (RA) to study gene expression. An additional cohort of 12 animals was used to isolate ECs from AA and DT, where sufficient material was obtained to study protein expression. PCR primers for genes of interest are listed in Table S1. Quantitative real time PCR was performed using LightCycler FastStart DNA Master SYBR Green I on a LightCycler System (Roche Applied Science, Indianapolis, IN). Primary and secondary antibodies used for Western blotting are listed in the expanded methods.

Statistical significance was assessed for the gene and protein expression ratios using one sided one-sample Wilcoxon test.

## RESULTS

Differential gene expression analysis identified 133 genes in athero-susceptible regions consistent with, and extending, our previously published work[7]; they included Connexin 43 (GJA1), identified as the most significantly upregulated gene in athero-susceptible ECs in agreement with previous work that showed higher Connexin 43 protein expression in flow dividers in rat arteries[18] and upregulated Connexin 43 mRNA expression in cultured endothelial cells that were subjected to disturbed flow *in vitro*[19]. VCAM1 was also upregulated in athero-susceptible ECs, in agreement with previously published work[20] and its upregulation was confirmed by QRT PCR (Fig S2).

### ER stress-related gene expression is upregulated in ECs of athero-susceptible arterial regions

Three independent and unbiased bioinformatics analyses indicated the presence of ER stress in regions of athero-susceptibility. Gene Ontology terms of protein folding, endoplasmic reticulum and unfolded protein binding were identified by the analysis tool DAVID[15] to be over-represented functional categories of the 133 differentially expressed genes (Fisher's Exact p value = $10^{-6}$–$10^{-3}$; Table 1). Furthermore, Gene Set Enrichment Analysis (GSEA) identified upregulation of gene sets related to ER metabolism, unfolded protein binding, ubiquitin conjugation and proteasome degradation in susceptible regions (Table S2). By Ingenuity Pathway Analysis, 73% of the upregulated genes formed a tightly connected network of interactions with highly significant enrichment scores (Fisher's exact p value = $10^{-69}$ to $10^{-17}$) based on known gene-protein and protein-protein direct relations (Fig. S3). This

network contained multiple genes that function in protein synthesis, protein folding and post-translational modification as well as inflammation and apoptosis, cellular processes that have been linked to endoplasmic reticulum (ER) stress [21]. Collectively, the genomic analysis indicated prominent differences in endothelial ER stress in regions of athero-susceptibility.

A subset of the differentially expressed genes was functionally categorized into protein biosynthesis and related pathways (Table 2 and Table S3). Each category showed elevated expression in the endothelium of athero-susceptible regions:

**mRNA processing—**Genes that code for proteins of the spliceosome complex, which processes hetero-nuclear RNA to mRNA, were upregulated in the endothelium from athero-susceptible regions. These included heterogeneous nuclear ribonucleoproteins (hnRP) A/B, D, M and U, PTBP1 (hnRNP I) and splicing factor PQ (SFPQ), genes that have been shown to function in mRNA splicing to remove introns from pre-mRNA [22]; expression of hnRNP A1 has been determined to increase with stress [23]. Translation initiation (eIF3S2) and elongation (eEF1E1) genes, DEAD box polypeptide (DDX3) which interacts with eIF3 to promote translation [24] as well as deoxyhypusine synthase (DHPS) which encodes hypusine, an unusual amino acid only found in eukaryotic translation initiation factor 5A, were also upregulated [25]. Collectively, mRNA processing activity of ECs in susceptible arterial regions was increased.

**Protein folding—**Numerous genes that have crucial functions in protein folding and post-translational modifications had higher expression in ECs from susceptible regions. Transcripts for two critical protein folding enzymes were upregulated: Peptidylprolyl isomerase (PPID) catalyzes the cis-trans isomerization of peptide bonds and protein disulphide isomerase (PDIA4 and PDIA5) catalyzes the formation of disulfide bonds [26]. Molecular chaperones, which aid protein folding, were more highly expressed in susceptible regions. Hsp70 (HSPA4, HSPA5) and Hsp40 (DNAJB6, DNAJB9) families of molecular chaperones bind to the growing polypeptide chains to increase the efficiency of folding [27]. Chaperonins (CCT4A, CCT6, CCT8, TCP1) recognize misfolded proteins in the cytosol and because of their barrel-like structure, create a favorable environment for protein refolding [27]. Observed increased folding capacity in susceptible ECs was consistent with an adaptive response to higher protein synthesis and ER stress in cells isolated from athero-susceptible sites.

**Protein transport and quality control—**Proteins that are destined for secretion are transported into the ER through the SEC61 protein translocator (SEC61A1, SEC61B) for glycosylation and proper folding [28]. Calnexin (CANX) and calreticulin (CALR) are two lectins that are part of the ER quality control system that ensures proteins are properly folded before they exit. Higher expression of SEC61 and CANX/CALR genes indicated increased ER quality control machinery in susceptible ECs consistent with higher expression of protein folding genes in these regions.

**Protein degradation—**Accumulation of polypeptides that fail to acquire their native conformation can jeopardize cellular function; therefore, misfolded proteins are removed by ER-associated degradation (ERAD) in conjunction with the ubiquitin/proteasome system (POMP, PSMD12, UFD1L) [29]; the latter genes were upregulated in susceptible ECs providing evidence for a protective mechanism against the accumulation of misfolded proteins in these regions.

**ER lipid synthesis—**ER stress results in an increase in the size of the ER membrane in stressed cells (ER dilation) [30]. Sterol-C4-methyl oxidase (SC4MOL) and squalene epoxidase (SQLE), which catalyze the intermediate reactions of cholesterol synthesis from squalene [31],

were more highly expressed in susceptible ECs, consistent with their rate-limiting role in the synthesis of cholesterol [32], an important component of the ER membrane.

Collectively, these data show a coordinated and significant upregulation of endothelial genes related to protein synthesis, folding, quality control and degradation indicative of the presence of chronic ER stress in susceptible regions.

Overload of misfolded proteins triggers the heat shock response in the cytosol and the unfolded protein response in the ER (Fig 2A), also known as ER stress [33]. A hallmark of ER stress is the upregulation of HSPA5, known as Binding Protein or GRP78 [33]. HSPA5 transcript was upregulated in susceptible regions (Table 2). This result was confirmed by QRT-PCR and Western blot (Fig 2B) in a comparison of ECs from aortic arch (AA; susceptible) and descending thoracic aorta (DT; protected). The ER stress marker, SERP1 [34], was also more highly expressed in susceptible regions.

## Unfolded protein response pathway is partially upregulated in athero-susceptible endothelium of the aorta

ER stress triggers UPR signaling [30]. Further analysis of UPR was focused on comparisons of gene and protein expression in the endothelium from the athero-susceptible AA and athero-protected DT where sufficient cells were accessible to allow protein measurements.

UPR is an adaptive response that is regulated by three ER membrane-localized signal transducers: inositol requiring kinase 1 (IRE1α), protein kinase-like ER kinase (PERK) and activating transcription factor 6α (ATF6α) (Fig 2A)[30]. IRE1α and ATF6α gene expressions were upregulated in AA compared to DT (Fig 3A, 3B); PERK expression, however, was unchanged (Fig 3C). Protein expression of the three transducers in their inactive form was not different between the two regions (Fig 3). Activation of the UPR occurs when HSPA5 dissociates from the signal transducers to bind unfolded proteins. ATF6α 90 kDa protein translocates to the Golgi where it is cleaved, and subsequently to the nucleus where the active 50 kDa form binds ER stress element (ERSE) and induces the transcription of molecular chaperones and apoptosis-related genes. Significantly higher expression of the 50 kDa ATF6α (Fig 3A) in AA was consistent with increased chaperone gene expression in susceptible ECs (Table 2).

The release of HSPA5 from IRE1α leads to homodimerization and auto-phosphorylation. Phosphorylated IRE1α had 70% higher expression in AA compared to DT (Fig 3B). Both phosphorylated IRE1α via its endoribonuclease activity, and ATF6α via its ERSE binding, upregulate the transcription of the spliced form of X-box binding protein 1 (XBP1) which is translated into XBP1 transcription factor. XBP1 gene and protein expression was elevated in AA compared to DT (Fig 3B). XBP1 nuclear translocation induces its binding to the UPR response element (UPRE) which leads to the transcription of ER associated degradation (ERAD) genes that include transcripts of ubiquitin/proteasome, glycosylation, disulphide bond formation and lipid synthesis consistent with the endothelial transcript profile in the susceptible regions (Table 2).

Similar to IRE1α, PERK activation also involves homodimerization and auto-phosphorylation comprising the third UPR signaling pathway. In contrast to the other 2 branches, however, PERK gene and protein expression and phospho-PERK expression were unchanged in AA compared to DT (Fig 3C). Phospho-PERK phosphorylates serine 52 of the eukaryotic translation initiation factor 2α (eIF2α) causing translational attenuation of most proteins with the exception of activating transcription factor 4 (ATF4). Translated ATF4 protein translocates to the nucleus where it binds to the UPRE. Neither phospho-eIF2α nor ATF4 protein expression was different between AA and DT, a finding consistent with inactivity of the PERK branch of

UPR (Fig 3C). However, ATF4 mRNA expression was higher in AA compared to DT (Fig 3C) indicating translational control of ATF4 expression.

The presence of ER stress was demonstrated in another athero-susceptible arterial region. ECs from athero-susceptible renal branch (RB) had significantly higher expression of HSPA5, ATF4 and XBP1 transcripts compared to ECs from athero-protected renal artery (RA) (Fig 4), providing further evidence for the presence of chronic ER stress in multiple athero-susceptible regions.

Activation of both ATF6α and PERK branches of UPR induces the expression of pro-apoptotic transcription factor CHOP. PERK induces the expression of anti-oxidative stress response genes [30, 35] by phosphorylating nuclear factor erythroid 2-like 2 (NRF2), a transcription factor that upregulates anti-oxidative genes [35]. Although CHOP transcript expression was more than two fold increased in AA compared to DT, NRF2 transcript expression was not changed (Fig 5), a finding in agreement with the lack of activation in the PERK branch of UPR. CHOP protein expression, however, was unchanged in AA compared to DT. Increased ATF4 and CHOP gene expression but not protein expression indicates an endothelial phenotype that is primed for further activation of the PERK branch of UPR. Furthermore, although CHOP transcript had higher expression in susceptible ECs, several anti-apoptotic genes that may mitigate apoptosis were also upregulated (Table S4). For example, ARMET, which inhibits ER-stress induced cell death [36], had significantly higher transcript expression in susceptible ECs (Table S4). Similarly, API5[37], BIRC2[38] and NIFL3[39], which have been shown to inhibit apoptosis in other cells as well as ECs, were also upregulated in susceptible regions. In addition to apoptotic balance, pro- and anti-inflammatory genes were also co-expressed in susceptible ECs (Table S4) providing further evidence for a steady-state phenotype of susceptible ECs primed for pathological change [7].

In summary, these data demonstrate the activation of adaptive UPR through ATF6α and IRE1α in response to chronic ER stress in susceptible ECs. In contrast, PERK, the third regulatory branch of UPR appears not to be involved in this adaptive response. Adaptive UPR signaling provides protection from the accumulation of misfolded proteins by selectively upregulating the protein folding and quality control mechanisms to cope with the protein overload in susceptible ECs.

## DISCUSSION

Heterogeneity in the functional state of the endothelium likely plays a key role in the spatial arterial susceptibility to atherosclerosis. Here, we have demonstrated the presence of ER stress as a signature for athero-susceptible endothelial phenotype in multiple arterial regions from a large cohort of animals from different backgrounds. While inter-animal variation is amplified in this approach, emergent findings are likely to reflect those in a general population. We report that endothelial phenotypes exhibiting chronic ER stress-related gene and protein expression characterize regions more susceptible to atherogenesis. Adaptive UPR signaling at these sites may mitigate localized stress effects by preventing the accumulation of pathological misfolded proteins.

UPR is a three pronged signaling network designed to maintain ER homeostasis by relieving the cells from accumulation of unfolded proteins. If the unfolded protein overload is not cleared, terminal UPR activates apoptosis. In acute UPR, eIF2α is rapidly phosphorylated to inhibit translation. On the other hand, adaptive UPR occurring over an extended period *in vivo* increases the protein folding and processing capacity of the ER [40]. Rutkowski and Kaufman [40] proposed that suppression of PERK and activation of ATF6α may occur in adaptive UPR. In agreement with their proposal, we present *in vivo* evidence for the activation of adaptive

UPR in susceptible ECs through ATF6α and IRE1α but not PERK. In such a scenario, additional risk factors may be required to trigger PERK activation. Many studies suggest that eIF2α phosphorylation, as a result of PERK activation, leads to increased ATF4 expression[30]; however, ATF4 mRNA, but not protein, expression was increased in AA compared to DT despite equivalent eIF2α phosphorylation. CHOP mRNA and protein expression had a similar profile to ATF4 in these regions. It is plausible that ATF4 and CHOP protein expression in nuclear protein extracts is different between the two regions; however, small number of ECs isolated from these regions did not allow for enough nuclear protein for quantitative measurements. Nuclear extracts pooled from 10 animals did not provide conclusive results (Fig S4). In response to pharmacologically-induced mild ER stress in mouse embryonic fibroblasts *in vitro* higher ATF4 and CHOP protein degradation rates have been reported compared to other UPR proteins [41]. The same study also demonstrated PERK activation in the apparent absence of eIF2α phosphorylation; similarly we observed a modest (16%) but non-significant increase in PERK phosphorylation (Fig 3C). This agreement between *in vivo* and *in vitro* results suggests a role for translational control of ATF4 and CHOP mRNA, the availability of which may provide susceptible ECs with a response mechanism by stabilizing their protein products to cope with acute increases in misfolded protein load as a result of local increases in pathological factors. The coexistence of pro-apoptotic CHOP expression and anti-apoptotic transcripts in susceptible regions suggests endothelial priming of, but not commitment to, apoptosis. A similar mechanism has been reported in macrophages during atherosclerosis where macrophage UPR was activated in the absence of apoptosis in early atherosclerotic lesions. However, apoptotic cells were apparent in advanced lesions in addition to further activation of UPR [42].

The initiation and progression of atherosclerosis involves inflammatory elements [43]. Low-grade chronic inflammation, as indicated by leukocyte accumulation in the intima was shown in the athero-susceptible region of the normal mouse aortic arch compared to more resistant regions [44]. Growing evidence links ER stress and inflammation [21]. Endothelial inflammation was shown to be UPR-mediated via XBP1 and ATF4 upregulation [45] and UPR activation in macrophages has been documented in early and advanced atherosclerotic lesions in apoE-deficient mice[42]. ROS generation due to protein folding provides the strongest link between inflammation and ER stress. The second link is via the IRE1α signaling molecule. Pro-inflammatory transcription factor NF-κB was shown to be activated by the association of IRE1α and adaptor protein TNF receptor-associated factor 2 (TRAF2) [46], although the details are not well understood. The third link is the phosphorylation of eIF2α, which inhibits translation, thereby repressing the synthesis of NF-κB inhibitor IκB. However, our previous study showed that NF-κB was not activated in swine AA or DT regions [7] suggesting that adaptive UPR does not induce NF-κB activation in normal animals in the absence of additional risk factors. Atherosclerotic risk factors, oxidized phospholipids [45, 47], oxidized LDL [48] and homocysteine [49] induce ER stress and upregulate the UPR pathway in ECs, suggesting that prolonged exposure to systemic risk factors can trigger the terminal UPR.

Athero-susceptible regions *in vivo* correlate with the presence of complex hemodynamics. Athero-susceptible disturbed flow triggers ER stress in ECs *in vitro*. Feaver *et al*.[50] provided *in vitro* evidence for the induction of HSPA5 expression by athero-susceptible flow through p38 activation. HSPA5 upregulation triggered the UPR pathway via ATF6α in cultured ECs. These *in vitro* studies provide evidence for a direct role of disturbed flow-induced ER stress in ECs that are consistent with the *in vivo* genomic and protein analyses presented here, although we cannot presume that the hemodynamics are the single most important determinant *in vivo*. While this manuscript was under review, a study demonstrating the increased expression of XBP1 at branch points of mouse aorta was published [51]. The study also showed that the overexpression of spliced XBP1 contributed to neointima formation in an artery

isograft model. Taken together, the current study and studies by Feaver et al.[50] and Zeng et al.[51] indicate a role for ER stress in the susceptibility to atherosclerosis.

Activation of UPR has been observed in disease pathogenesis [33] and in normal immune cells and hepatocytes whose function is to produce and secrete proteins [40]. However, this is the first study that demonstrates a steady state link between endothelial ER stress and disease susceptibility in healthy animals under physiological conditions. We propose ER stress/UPR to be an *in vivo* signature for athero-susceptible endothelial phenotype in which chronic low-grade stress primes the cells for pathological change. It will be important to understand the molecular mechanisms of adaptive UPR and the threshold signaling for terminal UPR to devise effective preventative therapies for atherosclerosis; our model can serve as an experimental system to study adaptive UPR under controlled manipulations. Recent studies identify antioxidants and chemical chaperones that reduce ER stress *in vitro* 52 and *in vivo* 53, respectively. A therapeutic role for these molecules to increase the protein folding capacity to reduce ER stress in ECs may be a viable therapy for atherosclerosis occurring in a variety of arterial vessels.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Non-standard Abbreviations and Acronyms

**DAVID**
: Database for Annotation, Visualization and Integrated Discovery

**EC**
: Endothelial cell

**ER**
: Endoplasmic reticulum

**ERAD**
: ER associated degradation

**ERSE**
: Endoplasmic reticulum stress element

**UPR**
: Unfolded protein response

**GSEA**
: Gene Set Enrichment Analysis

**IPA**
: Ingenuity Pathway Analysis

**MIAME**
: Minimum Information About a Microarray Experiment

**PaGE**
Patterns of Gene Expression

**UPRE**
UPR response element

## Gene Names

**ACSL4**
Acyl-CoA synthetase long-chain family member 4

**ATF4**
Activating transcription factor 4

**ATF6α**
Activating transcription factor 6 alpha

**ANKRD45**
Ankryin repear domain 45

**API5**
Apoptosis inhibitor 5

**ARMET**
Arginine-rich, mutated in early stage tumors

**ASB8**
Ankyrin repeat and SOCS box-containing 8

**BIRC2**
Baculoviral IAP repeat-containing 2

**CALR**
Calreticulin

**CALX**
Calnexin

**CALU**
Calumenin

**CCT**
Chaperonin containing T-complex polypeptide

**CHOP**
C/EBP homologous protein

**CNIH**
Cornichon

**DDX3X**
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked

**DHPS**
Deoxyhypusine synthase

**DNAJ**
Heat-shock protein 40 kDa

**EEF1E1**
Eukaryotic translation elongation factor 1 epsilon 1

**eIF2α**
eukaryotic translation initiation factor 2α

**EIF3S2**
Eukaryotic translation initiation factor 3, subunit 2 beta

**GJA1**
gap junction protein, alpha 1, 43kDa

**GRP78**
Glucose-regulated protein 78 kDa

**IRE1α**
Inositol-requiring enzyme 1 alpha

**NFIL3**
Nuclear factor, interleukin 3 regulated

**NRF2**
Nuclear factor erythroid 2-like 2

**HNRN**
Heterogeneous nuclear ribonucleoprotein

**HSPA**
Heat-shock 70 kDa protein

**HSPH1**
Heat-shock 105/110 kDa protein 1

**HUWE1**
HECT, UBA and WWE domain containing protein 1

**KPNA2**
Karyopherin alpha-2

**NUCB2**
Nucleobindin 2

**PERK**
Protein kinase-like ER kinase

**POMP**
Proteasome maturation protein

**PPAP2A**
Phosphatidic acid phosphatase type 2A

**PSMD12**
Proteasome 26S subunit, non-ATPase, 12

**PTBP1**
Polypyrimidine tract binding protein 1

**PDIA**
Protein disulphide isomerase

**PPID**
Peptidylprolyl isomerase

**RPN1**
Ribophorin I

**SAR1B**
SAR1 gene homolog B

**SC4MOL**
Sterol-C4-methyl oxidase-like

**SEC61**
SEC61 complex

**SERP1**
Stress-associated endoplasmic reticulum protein 1

**SFPQ**
Splicing factor proline/glutamine-rich

**SPPL2A**
Signal peptide peptidase-like 2A

**SQLE**
Squalene epoxidase

**SURF4**
Surfeit 4

**SYNCRIP**
Synaptotagmin binding, cytoplasmic RNA interacting protein

**TPST1**
Tyrosylprotein sulfotransferase 1

**TRAF2**
TNF receptor-associated factor 2

**UFD1L**
Ubiquitin fusion degradation 1 like

**VCAM1**
Vascular cell adhesion molecule 1

**XBP1**
X-box binding protein 1

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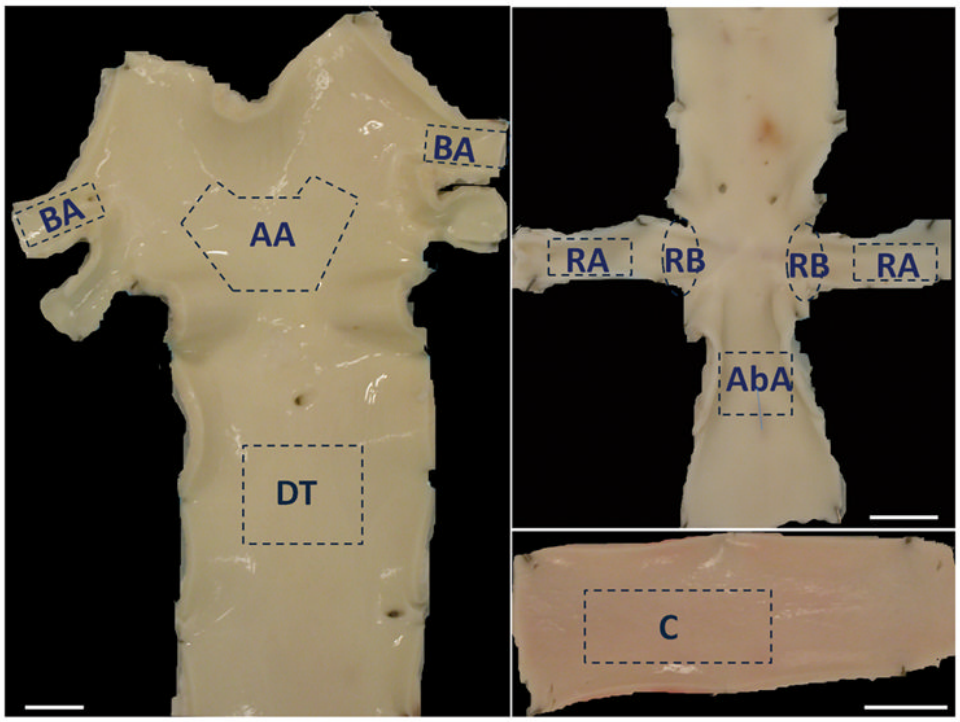
A




B

| Arterial Site Location | | |
|---|---|---|
| | **Athero-susceptible Regions** | **No. of samples** |
| **AA** | Aortic Arch: 1-cm$^2$ susceptible region located at the inner-curve and lateral walls | 8 |
| **BA** | Brachiocephalic Artery: 1-cm$^2$ susceptible region at the origin of the artery | 8 |
| **RB** | Renal Branch: 0.5 cm of proximal susceptible region of the renal artery | 8 |
| **Ab A** | Abdominal Aorta: 1-cm$^2$ susceptible region 2 cm distal to the branching of the renal artery | 8 |
| | **Athero-protected Regions** | |
| **C** | Carotid Artery: 1 cm$^2$ protected region in the straight section of the artery | 7 |
| **DT** | Descending Thoracic Aorta: 1-cm$^2$ protected region located from an undisturbed flow region of the dorsal descending thoracic aorta | 8 |
| **RA** | Renal Artery: 1 cm-long protected region 1.5 cm distal to the renal artery ostium | 8 |

**Figure 1.**
Arterial regions of endothelial isolation. ECs were gently scraped from multiple athero-susceptible and athero-protected regions for transcript and protein analysis. Representative images showing regions of isolation. Scale bar = 1cm.

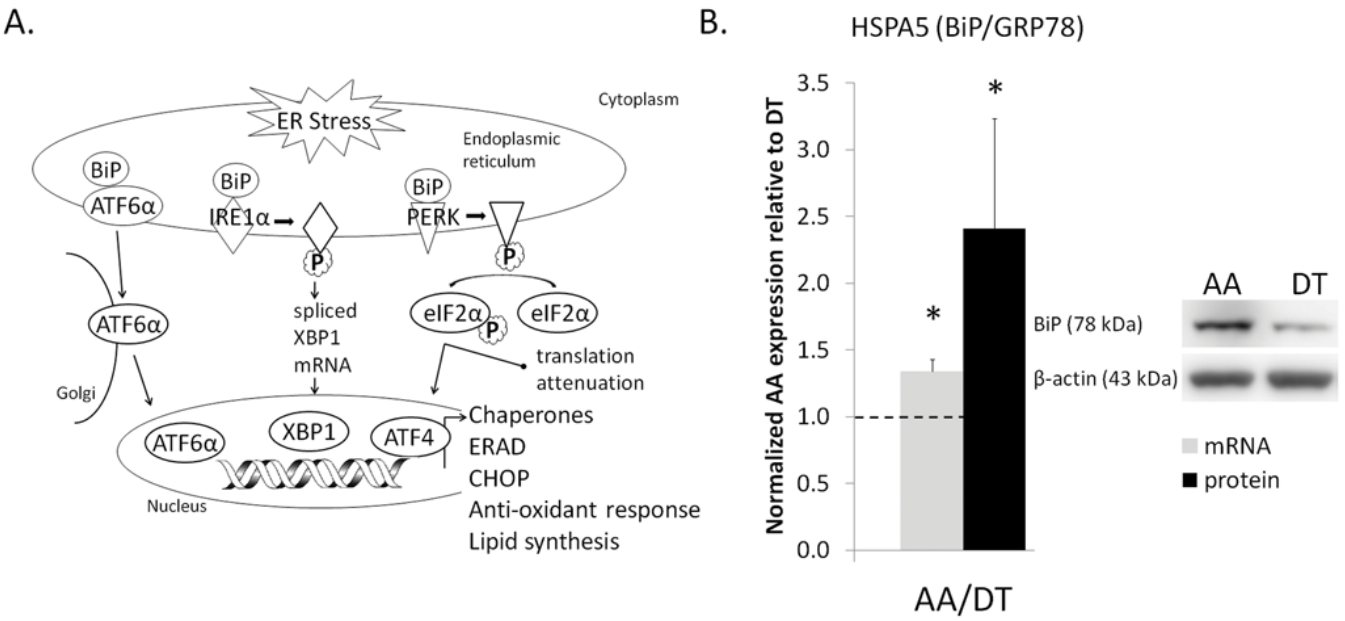



**Figure 2.**

ER stress and UPR. (A) UPR signaling outline (figure adapted from [30]). (B) ER stress marker HSPA5 gene and protein (78 kDa) expression in aortic arch, AA, normalized to descending thoracic aorta, DT, 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 ±SEM.*$p \leq 0.05$ one-sample, one sided, paired Wilcoxon test.

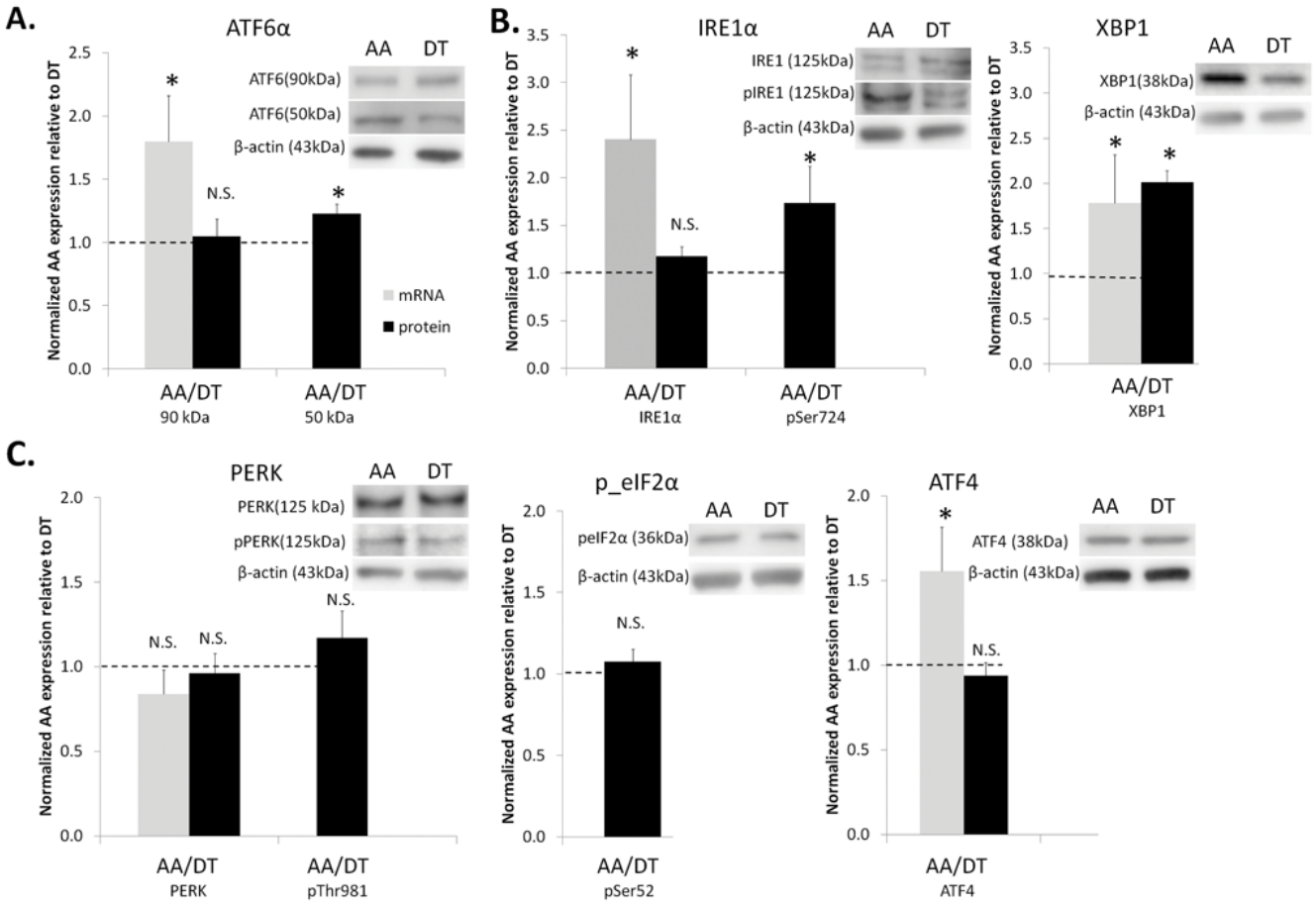



**Figure 3.**

UPR expression in arterial endothelium. Gene and protein expression of tripartite branches of UPR signaling: (A) ATF6α branch (B) IRE1α branch (C) PERK branch in aortic arch, AA, normalized to descending thoracic aorta, DT, for each paired sample based on their animal origin. Gene (n=6–10 paired samples) and protein (n=10–12 paired samples) expression was normalized to GAPDH and β-actin, respectively. Values > 1.0 indicate higher expression in AA. Data represent mean±SEM. N.S.: non-significant. *p≤0.05 one-sample, one sided, paired Wilcoxon test.

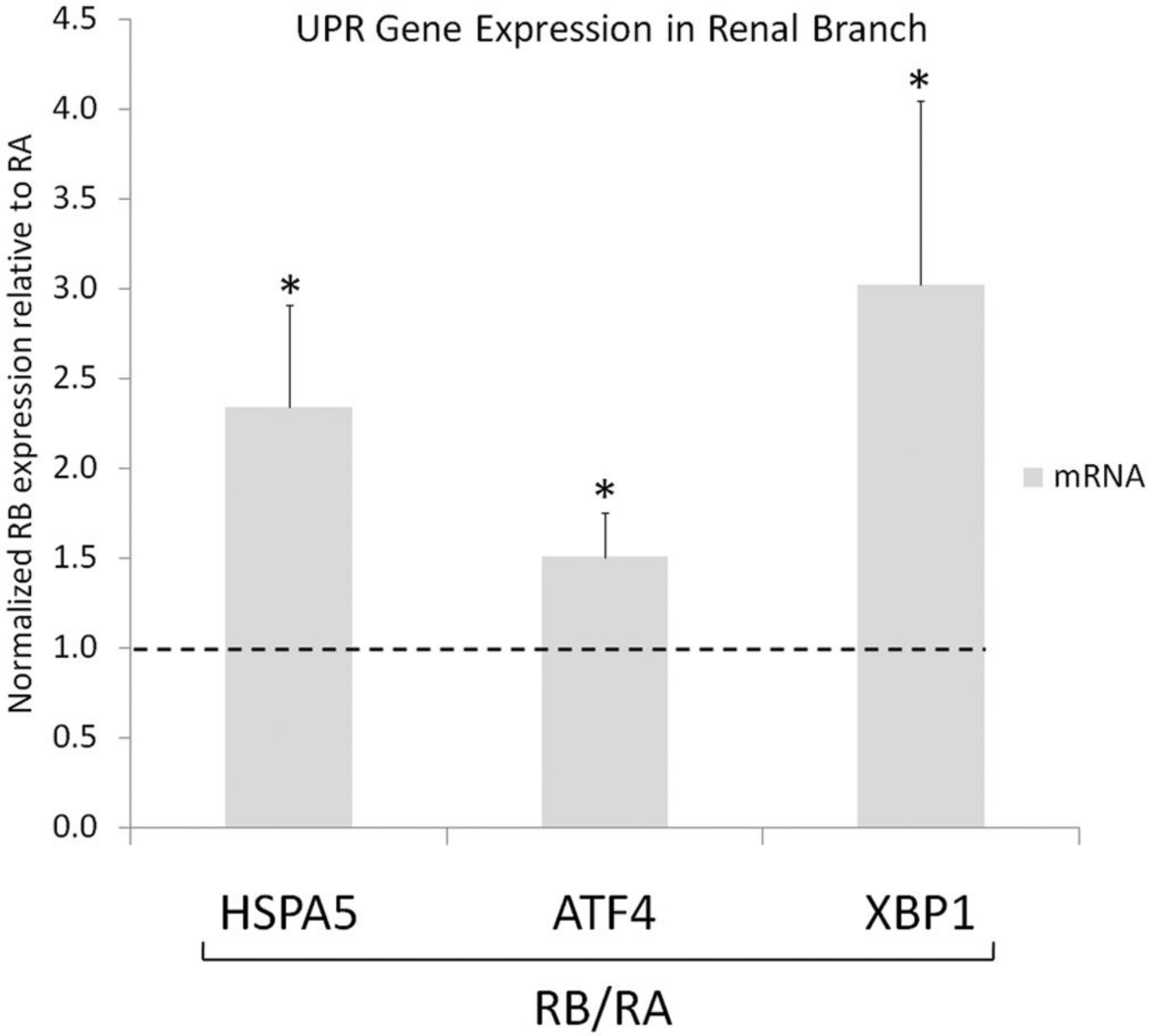



**Figure 4.**

UPR gene expression at renal branch and renal artery. HSPA5, ATF4 and XBP1 transcript in renal branch, RB, normalized to renal artery, RA, for each paired sample based on their animal origin. Gene expression (n= 6 paired samples) was normalized to GAPDH. Values >1.0 indicate higher expression in RB. Data represent mean±SEM. *p≤0.05 one-sample, one sided, paired Wilcoxon test.

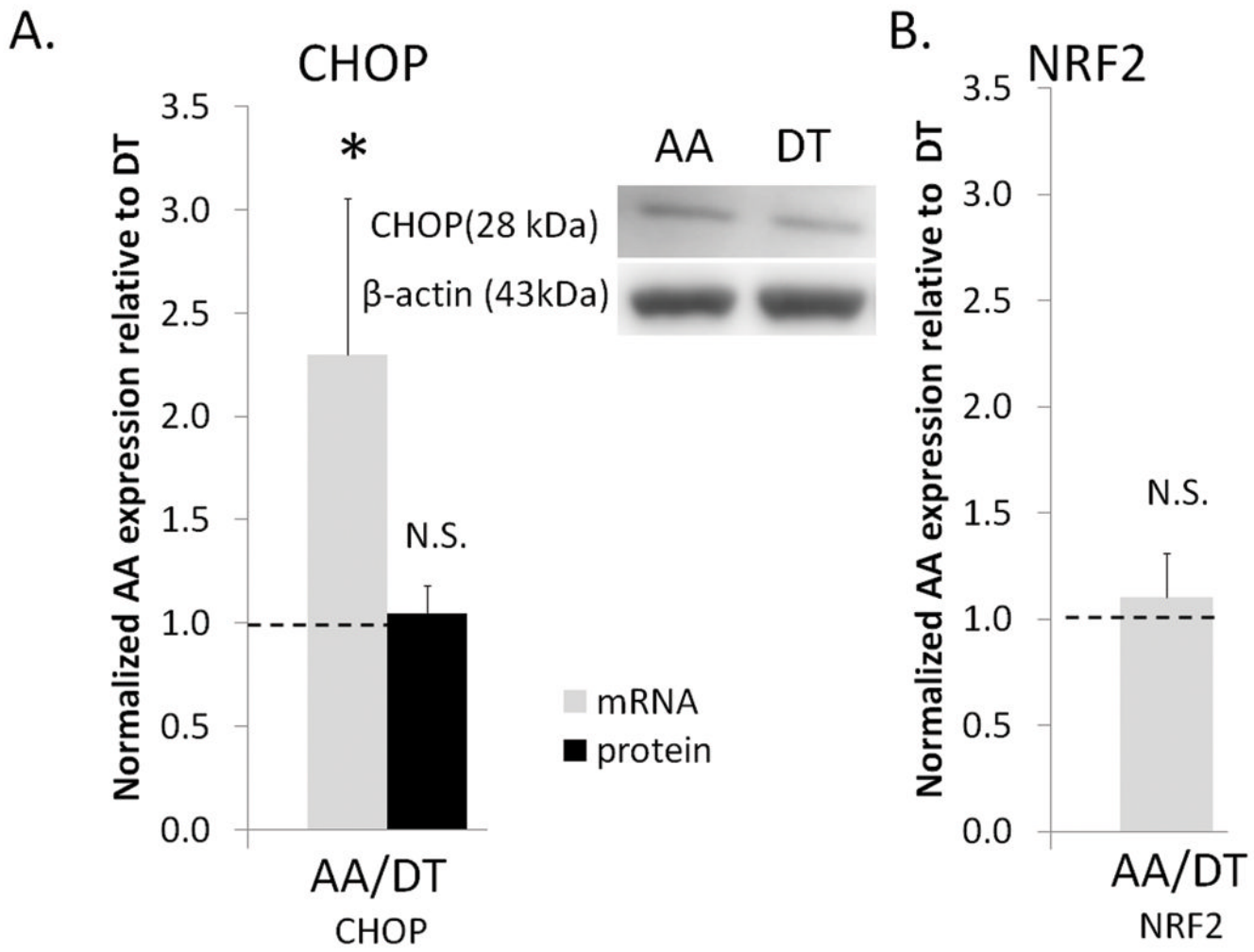



**Figure 5.**

Pro-apoptotic and anti-oxidative transcription factor expression in arterial endothelium. (A) Pro-apoptotic transcription factor CHOP gene (n=6 paired) and protein (n=12 paired) expression. (B) Anti-oxidative transcription factor NRF2 gene (n=5 paired) expression in aortic arch, AA, normalized to thoracic aorta, DT. Values > 1.0 indicate higher expression in AA. Data represent mean±SEM. N.S.: non-significant.*p≤0.05 one-sample, one sided, paired Wilcoxon test.