Network for Activation of Human Endothelial Cells by Oxidized Phospholipids

A Critical Role of Heme Oxygenase 1

Casey E. Romanoski, Nam Che, Fen Yin, Nguyen Mai, Delila Pouldar, Mete Civelek, Calvin Pan, Sangderk Lee, Ladan Vakili, Wen-Pin Yang, Paul Kayne, Imran N. Mungrue, Jesus A. Araujo, Judith A. Berliner, Aldons J. Lusis

Rationale: Oxidized palmitoyl arachidonyl phosphatidylcholine (Ox-PAPC) accumulates in atherosclerotic lesions, is proatherogenic, and influences the expression of more than 1000 genes in endothelial cells.

Objective: To elucidate the major pathways involved in Ox-PAPC action, we conducted a systems analysis of endothelial cell gene expression after exposure to Ox-PAPC.

Methods and Results: We used the variable responses of primary endothelial cells from 149 individuals exposed to Ox-PAPC to construct a network that consisted of 11 groups of genes, or modules. Further validating our method of network construction, modules were consistent with relationships established by cell biology studies of Ox-PAPC effects on endothelial cells. This network provides novel hypotheses about molecular interactions, as well as candidate molecular regulators of inflammation and atherosclerosis. We validated several hypotheses based on network connections and genomic association. Our network analysis predicted that the hub gene CHAC1 (cation transport regulator homolog 1) was regulated by the ATF4 (activating transcription factor 4) arm of the unfolded protein response pathway, and here we showed that ATF4 directly activates an element in the CHAC1 promoter. We showed that variation in basal levels of heme oxygenase 1 (HMOX1) contribute to the response to Ox-PAPC, consistent with its position as a hub in our network. We also identified G-protein–coupled receptor 39 (GPR39) as a regulator of HMOX1 levels and showed that it modulates the promoter activity of HMOX1. We further showed that OKL38/OSGN1 (oxidative stress–induced growth inhibitor), the hub gene in the blue module, is a key regulator of both inflammatory and anti-inflammatory molecules.

Conclusions: Our systems genetics approach has provided a broad view of the pathways involved in the response of endothelial cells to Ox-PAPC and also identified novel regulatory mechanisms. (Circ Res. 2011;109:e27-e41.)

Key Words: endothelial cells ■ phospholipids ■ gene expression ■ heme oxygenase ■ gene networks ■ genetics ■ genome-wide association studies

E levated levels of circulating low-density lipoprotein (LDL) result in the retention of LDL in the vessel wall, where the LDL undergoes aggregation and oxidative modification.1 Oxidized LDL has inflammatory activities that are strongly regulated by oxidized phospholipids2,3 and that have been shown to be present in human and mouse atherosclerotic lesions, where inflammation is increased,4 and in apoptotic cells, a component of atherosclerotic lesions.3 Plasma levels of specific phospholipid oxidation products of palmitoyl arachidonyl phosphatidylcholine (Ox-PAPC), reacting with the antibody EO6, have been shown to be prognostic for the development of atherosclerosis.3

Global gene expression studies of the responses of endothelial cells (ECs) to Ox-PAPC have shown that hundreds of genes are affected and that the affected genes show little overlap with those of classic innate immune responses such as nuclear factor-κB activation5 or other characterized inflammatory pathways. The 1-palmitoyl-2-(5,6)-epoxyisoprostane E2-sn-glycero-3-phosphocholine (PEIPC) and 1-palmitoyl-2-oxyvaleryl-sn-glycero-3-phosphorylcholine (POVPC) components of

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Correspondence to Aldons J. Lusis, PhD, 675 Charles E. Young Dr, MRL #3730, Los Angeles, CA 90095 (E-mail jlusis@mednet.ucla.edu); or Casey E. Romanoski, PhD, 9500 Gilman Dr, CMMW #219FF, La Jolla, CA 92093 (E-mail casey.romanoski@ucla.edu).

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Ox-PAPC have been shown to mimic most of the effects of Ox-PAPC. Several inflammatory functions regulated by Ox-PAPC have been described, including induction of a number of proinflammatory cytokines, inhibition of lipopolysaccharide action, induction of heme oxygenase 1 (HMOX1),2,3 and activation of the sterol biosynthetic pathway, procoagulant pathways, and the unfolded protein response pathway. Some of the functions of Ox-PAPC are antiinflammatory, whereas others are proinflammatory. Although aspects of the receptor regulation and signaling mechanisms of Ox-PAPC have been described, it is important to obtain an integrated view of the Ox-PAPC and signaling mechanisms of Ox-PAPC are antiinflammatory, whereas others are proinflammatory. Although aspects of the receptor regulation and signaling mechanisms of Ox-PAPC have been described, it is important to obtain an integrated view of the Ox-PAPC network to effectively target the induced pathways.

We reported previously that ECs from different strains of mice6 or from different heart transplant donors7 showed striking differences in the responses to Ox-PAPC, in part because of genetic variation. In the present study, to globally define the relationships among the molecules that respond to Ox-PAPC treatment in ECs, a systems genetics approach was used. Systems-based approaches attempt to examine biological processes more broadly than traditional reductionist approaches, which focus on individual genes or pathways. They often use high-throughput technologies such as expression arrays that generate genome-wide data. Data are then examined by statistical and modeling methods such as biological networks, in which the components of the systems are represented as “nodes” and their interactions as “edges.” In “systems genetics,” natural variations are used to perturb this system to allow modeling of the interactions. A fundamental concept of systems biology is that the whole is greater than the sum of the parts, and by examining the system as a whole, intrinsic novel properties may be identified that could not be derived linearly from the additive effects of the individual components.

For the present studies, we took advantage of naturally occurring gene expression variation in the human population. Global gene expression levels in early-passage EC cultures from 149 different heart transplant donors were examined with and without treatment with Ox-PAPC for 4 hours. An unbiased coexpression algorithm that models a scale-free gene network of the most responsive genes identified 11 modules of highly correlated genes that were enriched for a diverse array of novel functions. Analysis of this network revealed previously unknown effects of Ox-PAPC, most prominently an inhibition of cell division. Genes not previously known to be major regulators of Ox-PAPC action were identified as hub genes in modules, and new aspects of their regulation are reported. Follow-up validation studies focused on HMOX1, a known regulator of inflammation and a highly connected gene in one of the modules. Greater basal HMOX1 expression was associated with reduced responses of inflammatory genes to Ox-PAPC. A combination of network and genome-wide association studies (GWAS) revealed a regulatory role of G-protein–coupled receptor 39 (GPR39) in HMOX1 expression, which was confirmed by knockdown studies. Using promoter-reporter constructs for HMOX1, we further showed that GPR39 controls the transcription of HMOX1 through a particular promoter region. We also examined other network-based hypotheses. For example, we showed that ATF4 (activating transcription factor 4) directly regulates the expression of CHAC1 (cation transport regulator homolog 1), as predicted by the network model, and identified a specific regulatory element. On the basis of the network analysis, we identified OKL38/OSGIN1 (oxidative stress–induced growth inhibitor) as the major hub gene in the blue module and in subsequent studies determined that this gene regulates both basal and Ox-PAPC–induced levels of important inflammatory and antiinflammatory genes. The elucidation of the Ox-PAPC network and the discovery of novel pathways induced by Ox-PAPC in ECs by this network will be useful for formulating hypotheses to investigate the role of oxidized phospholipids in atherosclerosis.

Methods

Cell Culture and Treatments

A total of 158 human aortic endothelial cell (HAEC) cultures were isolated from aortic explants of 149 unique heart transplant donors in the University of California at Los Angeles (UCLA) transplant program and grown to confluence in 100-mm dishes as described previously.8 Nine of these cultures were duplicates (see expanded Methods in the Online Data Supplement, available at http://circres.ahajournals.org). We have previously validated our method of EC isolation on multiple cultures. We demonstrated that >95% of the cells were factor 8 positive and took up dil-acetyl LDL.8 In more recent studies, we have demonstrated in multiple EC preparations that >95% of the cells were positive for platelet endothelial cell adhesion molecule (data not shown). At 90% to 100% confluence, cells were treated in duplicate with either M199 media (Mediatech, Manassas, VA) containing 1% FBS (HyClone; Thermo Scientific, Logan, UT) or additionally with 40 μg/mL Ox-PAPC. For PEIPC experiments, treatment media contained either 5 μg/mL PEIPC or 50 μg/mL Ox-PAPC. Production of Ox-PAPC and synthesis of PEIPC have been described previously.9,10 We previously determined that the level of Ox-PAPC in the vessel wall of hypercholesterolemic rabbits is approximately 10 times the concentration used in the present studies.11 mRNA was harvested after 4 hours of treatment.

Gene Expression Profiling

Cytoplasmic RNA was hybridized to Affymetrix HT-HU133A microarrays (Affymetrix, Santa Clara, CA) as described previously.12 A total of 629 arrays were used to quantify gene expression, which corresponds to 158 cultures and 149 unique donors (Online Methods). Intensity values were normalized with the robust multiarray average normalization method as described previously.12 For the PEIPC experiment, Illumina (San Diego, CA) HumanRef-8v2 microarrays were used according to the manufacturer’s protocol, and data were normalized in Bead Studio (Illumina) by the rank invariant method. Array data are available in Gene Expression Omnibus accessions GSE30169.
Network Construction and Visualization
Duplicate expression measurements were averaged per condition and per donor. The 2000 most Ox-PAPC–regulated genes were identified by comparing the untreated and Ox-PAPC–treated values across the population with a paired t test. The 2000 most Ox-PAPC–regulated genes corresponded to those with t test probability values <1.0e-28. Two thousand transcripts were used so that the network would be inclusive of the most significant genes that were differentially expressed in response to Ox-PAPC, yet small enough to be visualized. Average transcript measurements for the 2000 transcripts in both conditions, untreated and treated with Ox-PAPC, were used in network construction. The pairwise adjacency matrix between genes was used to determine topological overlap between gene pairs.13,14 The topological overlap matrix was raised to the power of 12 to emphasize the difference between transcripts with many connections and transcripts with few connections. The topological overlap matrix was used to identify modules of highly coexpressed genes, and the clustering dendogram of the topological overlap matrix was cut by the dynamic hybrid method to define modules.15 Eleven modules were identified and arbitrarily named by colors. The gray module contained genes with dissimilar expression patterns and was not considered a true module. All of these analyses were performed in R with the Weighted Gene Coexpression Network Analysis (WGCNA) package.16 Network visualization was performed in Cytoscape version 2.6.3.18 For details of the network visualizations, refer to the Online Methods.

Genotyping and Association Analysis
Genomic DNA was isolated from HAECs with the DNeasy kit with RNase treatment (Qiagen, Valencia, CA). For HMOX1 microsatellite genotyping, an FAM-labeled polymerase chain reaction product was generated with the primers 5' GAG CCT GCA GCT TCT CAG AT 3’ and 5’ ACA GCT GAT GCC CAC TTC CT 3’. Microsatellite alleles were then classified as “short” (<29 repeats) or “long” (≥29 repeats). Single-nucleotide polymorphism (SNP) genotypes were acquired as described previously12 (Online Methods). The 147 unique donors with complete gene expression and genotyping data were used in association analysis. Genotype-gene expression associations (ie, expression quantitative trait loci) were tested by linear regression with the “linear” option in PLINK 1.4.17

Quantitative Reverse Transcription–Polymerase Chain Reaction, Western Blotting, and Small Interfering RNA Knockdown
Quantitative reverse transcription–polymerase chain reaction was performed with the Roche LightCycler 480 (Roche Diagnostics, Indianapolis, IN). Experiments with small interfering RNA (siRNA) were performed as described previously.18 siRNAs used in the present study were HMOX1 siRNA #1 (Qiagen catalog No. SI00030389), HMOX1 siRNA #2 (Qiagen catalog No. SI00030396), GPR39 siRNA #1 (Qiagen catalog No. SI00430416), GPR39 siRNA #2 (Qiagen catalog No. SI00430423), GRID1 siRNA #1 (Qiagen catalog No. SI00430836), and GRID1 siRNA #2 (Qiagen catalog No. SI00430843). OKL38 siRNAs were Qiagen SI 00665182 and SLE6650196.

Heme Oxygenase Activity Assay and Protein Quantification
Heme oxygenase activity was measured by bilirubin generation as described previously19 (Online Methods).

Luciferase Constructs and Assays
The CHAC1 promoter and deletion constructs were generated with polymerase chain reaction from a fosmid (BAC/PAC clone No. G248P8704A5) and cloned into pGL3Basic (Promega, Madison, WI). The HMOX1 luciferase constructs were described previously (−4.5 kb= pHOGL3/4.5 and −9.4 kb= pHOGL3/9.4).

Results
Network Modeling Identifies Modules of Tightly Coexpressed Genes
We sought to obtain a systems-level view of the responses of ECs to Ox-PAPC to provide a framework for mechanistic studies. To achieve this, primary cultured HAECs (or the “system”) were exposed for 4 hours to either control medium or medium containing Ox-PAPC (40 μg/mL), and transcript levels of the Ox-PAPC–responsive genes (the “elements” of the system) were quantified with global expression arrays. This time period was chosen on the basis of previous time-course experiments to permit detection of a robust yet early response. The HAEC cultures obtained in the present study were not patients but heart transplant donors whose hearts were deemed healthy enough for transplantation by the transplant surgeons before tissue collection. All clinical information, including age and sex, remained anonymous in the present study (sex was independently determined from the X chromosome genotypes); however, the average age of the donors in the transplant program is known to be approximately 25 years. Clinical phenotypes such as use of medications and hormonal status were not known. Because most cells were used at passages 4 to 7, most acute drug and hormonal effects would likely be lost by this passage. Of approximately 19 000 genes analyzed, the 2000 transcripts most affected by Ox-PAPC (paired t test values <1e-28) across the population (see below) were used for network analysis.

Because Ox-PAPC is a mixture of several oxidation products of PAPC, we tested whether the Ox-PAPC response in HAECs was similar to that observed after treatment with the most potent single phospholipid component of Ox-PAPC: 1-palmitoyl-2–5,6-epoxyisoprostane E₂₅sn-glycero-3-phosphocholine (PEIPC).9 HAECs from a single donor were treated with 5 μg/mL PEIPC or 50 μg/mL Ox-PAPC. Gene expression arrays quantified the gene expression levels at baseline and after treatment, and the fold-change values were calculated for each transcript. A highly conserved response was observed between Ox-PAPC and PEIPC (P<2e-16). Furthermore, the average fold change for the 2000 genes from 149 donors used to construct the network was highly correlated with the fold change in these same genes by PEIPC in the donor tested (P<2e-16; Online Figure I). These data demonstrate that PEIPC has a very similar effect to Ox-PAPC.

To model an unbiased coexpression network, we took advantage of naturally occurring variations in the human population that perturbed individual gene expression patterns with and without Ox-PAPC treatment. We have previously shown that the transcriptomic responses to Ox-PAPC are determined largely by genetic factors.12 A coexpression network algorithm, WGNCA,15 based on “topological overlap” of gene expression both with and without Ox-PAPC treatment, was used to construct the network. Topological overlap is a metric of similarity that reflects the relationship

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strength between gene pairs. It depends on the correlation between genes, as well as shared correlations with other genes. The network was visualized with Cytoscape software (Figure 1A). In concordance with other biological networks, our coexpression network was modeled to exhibit a scale-free structure so that few genes had many connections and most genes had few connections.22,23

Genes were grouped into color-coded modules based on similarity in patterns of expression across the sample population. The network of endothelial genes most significantly induced by Ox-PAPC consisted of 11 modules of tightly connected genes (Figure 1; Online Figures II through XII). A full list of the transcripts included in the network analysis, arranged by modules in order of highest to lowest connectivity, are included in Online Table I. Multiple probe set IDs for the same gene on the HT-HG133A array were treated as independent transcripts, which is why more than 1 node may appear in a module with the same gene symbol. This approach was taken so that alternative transcripts would be maintained in the network.

Coexpressed Modules Are Enriched for Functional Pathways

Coexpression is evidence for coregulation and suggests involvement in similar cellular functions. To examine whether genes in a given module were in the same pathway, we tested for enrichment of each module based on Gene Ontology categories. Several modules exhibited enrichment for pathway ontologies (Table 1). These included novel as well as previously reported pathways that are affected by Ox-PAPC treatment. A particularly striking novel observation was seen in the turquoise module, which was highly enriched for the cell cycle genes that were downregulated after 4 hours of Ox-PAPC treatment (Figure 1B; Online Figure III). Another enriched pathway in the turquoise module not previously implicated in the response of ECs to Ox-PAPC treatment was nucleobase, nucleoside, nucleotide, and nucleic acid metabolism. Other modules/pathways not previously observed included endocytosis, enriched in the black module (Online Figure X), and Golgi organization in the brown module (Online Figure VII).

Molecular interactions determined by the network revealed novel relationships in previously reported Ox-PAPC-responsive pathways such as the unfolded protein response (UPR). The genes in the UPR pathway segregated into 2 modules, the purple and blue, which corresponded to the X-box binding protein 1 (XBP1; Online Figure IV) and activating transcription factor 4 (ATF4; Figure 2) arms of the UPR, respectively. Clustering of the first principal components of gene expression in each module showed that the blue and purple modules were most similar to each other relative to all other modules in the network, which suggests that these 2 modules share functional characteristics and capture biological regulatory mechanisms (Online Figure XIII).

Enrichment for sulfur amino acid metabolic processes and glutamine family amino acid metabolic processes in the blue module (Table 1) is consistent with the evidence that treatment of ECs with Ox-PAPC increases oxidative stress.24 In particular, enrichment for sulfhydryl proteins in the blue module supports the novel mechanistic hypothesis that covalent interaction of certain Ox-PAPC compounds with free sulfhydryl groups triggers the Ox-PAPC response.
PEIPC has been shown to covalently bind to proteins. Our studies suggest that the allylic epoxide group on PEIPC, which readily binds sulfhydryls, is most likely the major active site on this molecule. Furthermore, we identified modules that represented pathways previously reported as regulated by Ox-PAPC, including regulation of actin filament polymerization in the green module (Online Figure VI) and elevation of cytosolic calcium ion concentration in the red module (Online Figure VIII), which indicates the ability of coexpression network modeling to provide both novel and established relationships in the response of ECs to Ox-PAPC (Table 1; Figure 1B). Knowledge of the components and connectivity of these genes will be useful in identifying the signal transduction pathways that regulate these processes.

Hub Genes Are Likely to Be Key Regulators

In addition to large-scale functional enrichments, our network analysis identified hub genes that are potentially important regulators of major pathways activated in response to Ox-PAPC. One such gene is OKL38/OSGIN1, which was the most connected gene in the blue module (Figure 2). This gene was previously identified by us as a redox gene regulated by Ox-PAPC, but its pivotal role was not recognized. Supporting the hypothesis that OKL38 plays a key role, we have identified a number of inflammatory and antiinflammatory genes regulated by OKL38 in knockdown studies (Online Figure XIV). Two OKL38 siRNAs were used that achieved ~50% of OKL38 transcript levels (data not shown). We demonstrated that knockdown of OKL38 increased both basal and Ox-PAPC–induced levels of interleukin 8 (IL-8), ATF4, and KLF4 (Kruppel-like factor 4), all molecules previously associated with EC inflammatory responses. This is the expected result if the expression levels of these genes are controlled by increased oxidative stress and OKL38 protects against oxidative stress. In addition, we observed that knockdown of OKL38 dramatically increased both basal and Ox-PAPC–induced levels of HMOX1, which suggests

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GO ID indicates Gene Ontology identification number.

Table 1. Gene Ontology Enrichments in Modules of the HAEC Gene Coexpression Network
a close relationship between the levels of these 2 molecules. Two other members of the blue module showed no effect of OKL38 knockdown (data not shown).

Genes at GWAS Loci for Coronary Artery Disease Are Regulated by Ox-PAPC, Exhibit Regulation of Gene Expression, and Are in the HAEC Network

Next, we performed systems genetics analyses with the goal of elucidating the disease genes and pathways of atherosclerosis. To this end, we integrated results from a recent comprehensive GWAS analysis of coronary artery disease (CAD) with our endothelial systems data in 2 ways. First, we queried genes that resided under GWAS peaks and their local neighborhoods for regulation by Ox-PAPC in our HAEC coexpression network. Second, we identified genes whose expression exhibited regulation by the peak GWAS SNPs.

We used CAD GWAS loci identified by Schunkert and colleagues\(^27\) using 22,233 case subjects and 64,762 control subjects, with additional genotyping for peak loci in 56,682 additional individuals. For the present analysis, we began with 31 genes under 25 association peaks that were measured in our HAEC population (Online Table II). Five genes (LDLR [low-density lipoprotein receptor], NT5C2 [5-nucleotidase, cytosolic II], SH2B3 [SH2B adaptor protein 3], UBE2Z [ubiquitin-conjugating enzyme E2Z], and COL4A1 [collagen, type IV, alpha 1]) were regulated by
Ox-PAPC by an average of at least 1.1-fold across the HAEC population \((P<1e-21)\). Four of these genes were included in the HAEC network and resided in the blue (LDLR and UBE2Z), green (NT5C2), or brown (SH2B3) modules (Online Table II). To explore the network neighborhoods, we identified the transcripts most connected to these genes in our network and visualized these as “GWAS gene-centered subnetworks” of the overall network. An example is shown for NT5C2 in Online Figure XV. Taken together, these data provide evidence that not only are LDLR, UBE2Z, NT5C2, and possibly COL4A1 positional candidates for CAD, but these genes may also play a role in disease based on regulation by Ox-PAPC and their connectivities to other Ox-PAPC-regulated genes. LDLR is already known to play a major role in CAD, which serves as further evidence that regulation by Ox-PAPC is a valid approach for defining CAD genes.

To achieve our second systems genetics aim, we identified transcripts whose expression at baseline, after Ox-PAPC treatment, or whose Ox-PAPC fold induction exhibited regulation, and thus expression quantitative trait loci, at peak GWAS SNPs. In other words, we searched for genes whose expression may be the causal intermediate between genetic variation and CAD. Of the 16 GWAS SNPs genotyped in the HAEC data, 4 harbored an expression quantitative trait locus \((P<1e-6; \text{Online Table II})\). Three of these loci regulated expression traits that were distal\(\text{trans}\) to the GWAS locus. However, rs11556924 on chromosome 7q32.2 significantly \((P=7.8e-9)\) regulated baseline expression of KLHDC10 (kelch domain–containing 10), which is located within the locus. These data prioritize KLHDC10 for further investigation as a causal gene at the 7q32.2 locus for CAD.

Validation of Causal Interactions: CHAC1 Is Directly Regulated by ATF4

We previously identified the CHAC1 gene as a target of the UPR based on its position as a hub gene in a module enriched for UPR genes.\(^{7,28}\) CHAC1 is induced in response to UPR activators such as thapsigargin, and siRNA knockdown of several UPR genes, including ATF4, activating transcription factor 3, and CHOP (C/EBP homologous protein), reduced CHAC1 induction.\(^{28}\) CHAC1 is highly conserved, from bacteria to mammals, but before these studies, its function had not been established. Subsequently, we have shown that it has a key role in apoptosis (data not shown); therefore, the precise mechanism of its regulation is of considerable interest.

Our network modeling revealed that ATF4 and CHAC1 were connected by a particularly strong edge and that they had many shared neighbors (genes with shared edges; Figure 2). This suggested that ATF4 might directly regulate the expression of the CHAC1 gene. To address this question, we constructed a series of promoter constructs coupled to luciferase and tested them for activity after transfection of 293 cells (Figure 3). Cotransfection of ATF4 robustly induced the expression of the CHAC1 promoter, and this required an ATF/CREB site at −267 of the promoter (Figure 3). Additionally, ATF4 cotransfection was sufficient to activate CHAC1 transcription in sequential internal promoter-reporter deletion constructs, in which the −267 ATF/CREB site remained intact (Figure 3), and scrambling the −267 ATF/CREB site was sufficient to block the ATF4 effect on CHAC1 promoter activity (data not shown). Importantly, plasmid overexpression of activating transcription factor 3, spliced XBP1, or CHOP (data not shown) did not affect CHAC1 promoter activity. These data highlight the ATF4 interaction with CHAC1 via an ATF/CREB site as the major determinant of CHAC1 regulation. Additionally, these data indicate that activating transcription factor 3 and CHOP do not directly activate the CHAC1 promoter. This demonstrates the power of the network modeling to highlight functional gene interactions and provide important information on complex signaling networks that can be further refined by use of directed strategies.

In addition to wide-scale functional enrichments, the network recovered several known interactions. For example, in the blue module, the UPR transcription factor ATF4 was tightly coupled to its known targets, including asparagine synthetase,\(^{29}\) tribles homolog 3,\(^{30}\) and vascular endothelial growth factor A (VEGFA)\(^{31}\) (Figure 2). In addition NRF2/NFE2L2 (nuclear factor erythroid-derived 2-like 2) was located in the blue module with several of its known target genes, including NQO1 (NAD[P]H dehydrogenase quinone 1),\(^{32}\) GCLM (glutamate-cysteine ligase modifier subunit),\(^{32}\) and TXNRD1 (thioredoxin reductase 1).\(^{33}\) (Figure 2).

HMOX1, a Gene Previously Implicated in Protection Against Atherosclerosis, Affects Many Genes in the Network and Suppresses the Response of Inflammatory Genes to Ox-PAPC

Among the many hypotheses of gene-gene interactions generated by the network, we focused on the role of HMOX1\(^{34}\) and its regulation to illustrate the applications of the network model. We focused on this gene on the basis of the importance of HMOX1 in animal\(^{35–39}\) and human\(^{40–42}\) studies. HMOX1 itself is a “hub” located in the blue module (Figure 2), along with a number of genes previously identified as responsive to Ox-PAPC, including NQO1,\(^{24}\) NRF2/NFE2L2,\(^{32}\) ATF4,\(^{18}\) VEGFA,\(^{43}\) and LDLR.\(^{44}\)

There was marked baseline variation among 149 EC donors, with a 9-fold difference observed between extremes of HMOX1 basal expression (Figure 4A). In contrast, only a 2-fold difference among donors was observed in HMOX1 transcript levels in cells treated with Ox-PAPC. Taken together, these data suggest that there is a ceiling to the expression of HMOX1 in ECs in this model system. We verified that HMOX1 mRNA induction was closely reflected at the levels of protein and heme oxygenase enzymatic activity in HAECs and in HeLa cells (Online Figure XVI). These data confirm that HMOX1 mRNA expression is a suitable means for evaluation of HMOX1 activity and support the hypothesis that variation in its basal activity significantly impacts the Ox-PAPC response.

These findings led us to hypothesize that basal levels of the enzyme would protect against the effects of Ox-PAPC. To model a causal relationship whereby basal HMOX1 levels would influence the induction of genes by Ox-PAPC, we calculated the correlations between HMOX1 basal levels and the levels of genes in various pathways after treatment with
Ox-PAPC. Many of the key molecules that coordinate the aforementioned endothelial responses were strongly negatively correlated to baseline HMOX1 expression, including the adhesion molecule vascular cell adhesion molecule 1 \((P=3.0e-3)\) and genes regulated by the UPR, including VEGFA \((P<1.0e-10)\), activating transcription factor 3 \((P=5.0e-8)\), and the inflammatory cytokine interleukin-6 \((P=1.4e-6; \text{Figures 4B through 4E})\). When we examined the distribution of HMOX1-correlated genes in the overall network, we found that HMOX1-correlated genes were dispersed among all of the modules in the network (Figure 5A). These data support the hypothesis that basal HMOX1 modulates the Ox-PAPC response of genes from a diverse set of pathways. Although most genes were negatively correlated to basal HMOX1 (meaning greater basal HMOX1 corresponded to less Ox-PAPC-treated levels of the other transcript), exceptions were predominantly found in the red module (Figure 5A; Online Table III). Such positively correlated genes included VTI1B (vesicle transport through interaction with t-SNAREs homolog 1B; \(R=0.55, P=7.6e-15\)), ZMAT3 (zinc finger, matrin-type 3; \(R=0.52, P=5.6e-13\)), SOX4 (sex-determining region Y–box 4; \(R=0.50, P=8.2e-12\)), CERK (ceramide kinase; \(R=0.48, P=3.3e-11\)), and SESN1 (sestrin 1; \(R=0.48, P=6.0e-11\)). Unlike other genes in the red module, these molecules are not implicated in calcium signaling and have not been grouped together in a Gene Ontology category.

Given the importance of HMOX1 in animal\(^{35–39,45–47}\) and human\(^{48–50}\) studies and the observation that basal HMOX1 levels were more variable than post–Ox-PAPC treatment, we hypothesized that greater baseline HMOX1 levels would be protective against exposure to Ox-PAPC. To test this, we correlated individual HMOX1 basal levels with the degree to which each individual responded to Ox-PAPC. The number of genes regulated above a given threshold per individual was defined as responsiveness to Ox-PAPC. Baseline HMOX1 expression was highly correlated with individual responsiveness at thresholds above 2-fold, most significantly at the 7.5-fold threshold \((R=-0.57)\). Taken together, these data strongly support the hypothesis that HMOX1 plays a critical role in mitigating multiple cellular responses to Ox-PAPC.
To directly test the role of HMOX1 levels on target gene expression, the HMOX1 transcript was silenced with 2 different siRNAs to 10% to 15% of baseline values (Figure 5B). Expression of the proinflammatory cytokines interleukin-1β, interleukin-6, and monocyte chemotactic protein 1 was found to be more highly induced after HMOX1 knockdown than in the control (Figure 5C). Furthermore, the cholesterol regulatory genes LDLR and INSIG1 (insulin-induced gene 1) were also more highly induced in response to Ox-PAPC as a result of HMOX1 knockdown, which suggests that sterol regulation may depend on HMOX1 (Figure 5C).

**Regulation of HMOX1 Expression by NRF2**

In an effort to discover how basal HMOX1 transcript was regulated, we examined the relationship between HMOX1 and target genes.

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**Figure 4. HMOX1 variation and correlation with proinflammatory gene expression.** A, Gene expression intensity (y-axis, log2 scale) is shown for the HMOX1 transcript at baseline (solid black circles) and after treatment with Ox-PAPC (open red circles) in the HAEC population. A total of 149 donors are ranked along the x-axis according to baseline expression. B–E, Basal HMOX1 levels are plotted on the x-axes against the Ox-PAPC–treated levels of vascular cell adhesion molecule 1 (VCAM1) (B), VEGFA (C), activating transcription factor 3 (ATF3) (D), and interleukin (IL)-6 (E) on the y-axes. R and P values result from Pearson correlation. Data shown in Figure 4A are an expansion of data from 96 donors published previously in the same manner (Romanoski et al12).

**Figure 5. Regulation by HMOX1 in the network.** A, Node border color signifies module membership, and the fill indicates whether the Ox-PAPC–treated expression level of a gene was correlated with HMOX1 basal levels (red is negative and green is positive correlation). B, siRNA-mediated knockdown of HMOX1 is shown for 2 siRNAs with the scrambled control (Scr). C, The fold changes of interleukin (IL)-1β, IL-6, monocyte chemotactic protein (MCP)-1, LDLR, and INSIG1 were normalized to the fold changes measured in the scrambled control. mRNA was measured by quantitative reverse transcription polymerase chain reaction, and averages plus or minus SD are shown. Asterisks indicate P<0.05.
and NRF2, which was also in the blue module. NRF2 has been shown to activate HMOX1 transcription on translocation to the nucleus and binding to antioxidant response elements in the HMOX1 promoter.\textsuperscript{51} In the absence of Ox-PAPC treatment, NRF2 and HMOX1 were negatively correlated across the 149 donors ($R=-0.3$, $P=7.2e-5$), which means that greater NRF2 expression corresponded to less HMOX1 expression (Online Figure XVIIIA). Surprisingly, correlations between NRF2 and HMOX1 were not significant after exposure to Ox-PAPC, and no significant correlation existed between untreated NRF2 levels and treated HMOX1 levels (Online Figure XVII, B and C). These data were consistent with studies that demonstrated a role of other transcription factors regulating HMOX1 induction and cell type–dependent regulation by NRF2.\textsuperscript{52} However, NRF2 levels were highly correlated, at baseline and after Ox-PAPC treatment, with other known target antioxidant molecules, including NQO1, CCAAT/enhancer-binding protein beta (CEBPB), GCLM, TXNRD1, and glutathione and glucuronyl transferases (glutathione S-transferase kappa 1 and $\beta$-1,3-glucurononyltransferase 3; Online Figure XVIII). These observations confirmed that expression data from the present sample population were able to recover known molecular relationships and confirmed the role of NRF2 in oxidant gene regulation. Nonetheless, these data demonstrate that NRF2 levels explained only a small portion of HMOX1 basal regulation. These results suggest that mechanisms other than NRF2 modulate basal HMOX1 levels.

**HMOX1 Expression Does Not Exhibit Robust Local Genetic Regulation**

We investigated the possibility of basal HMOX1 transcript regulation by an HMOX1 promoter microsatellite polymorphism. This dinucleotide GT expansion has been associated with various inflammatory diseases, including CAD, in several populations (reviewed in Exner et al\textsuperscript{41}). In these studies, longer alleles of the (GT) repeat were associated with disease. The mechanism by which longer alleles convey risk has been suggested to occur through altered expression of HMOX1.\textsuperscript{40} Most convincing was the recent report by Taha et al,\textsuperscript{42} who showed that short microsatellite alleles produced more HMOX1 than long alleles and that short alleles were protective against oxidative stress in human umbilical vein ECs. To test for differences in HMOX1 expression as a function of promoter alleles in HAECs, we genotyped the (GT)$_{29}$ promoter microsatellite and associated the dichotomized genotypes [short alleles $<29$ (GT) repeats and long alleles $\geq29$ (GT) repeats, in concordance with previous studies\textsuperscript{41}] with HMOX1 expression levels. HMOX1 expression at baseline was associated with the microsatellite polymorphism in the whole population ($n=149$, $P=1.9e-3$) and in males ($n=113$, $P=1.9e-3$) but not in females ($n=36$, $P=0.533$; Online Figure XIX, top row). Where significant, HMOX1 expression was greater in individuals with more (GT) repeats. The fold change of HMOX1 induction was significant in the whole population ($P=2.4e-3$) and in males ($P=2.0e-3$; Online Figure XIX, bottom row). With regard to HMOX1 fold change on treatment with Ox-PAPC, individuals with longer alleles displayed lesser fold-change values. These results contradict the previous findings, which suggested that short alleles would produce more HMOX1.\textsuperscript{43}

Next, we examined SNPs located in or near HMOX1 to test for additional local regulatory variation. Sixty-two SNPs were genotyped with minor allele frequency $>5\%$ that were within 100 kb of the HMOX1 transcriptional start site on chromosome 22. Of these SNPs, the most significant association with the basal levels of HMOX1 was for rs5995385 ($P=9.0e-3$, minor allele frequency $=0.39$), downstream of HMOX1. After correction for the multiple SNPs tested in the local region, this association became insignificant, which suggests that no local SNPs significantly regulated HMOX1 basal expression.

Because we did not observe robust regulation of HMOX1 mRNA expression by local genetic variation, we hypothesized that variation at the HMOX1 locus may perturb HMOX1 function by other means. In particular, we focused on rs2071747, which is a missense mutation in the seventh amino acid of HMOX1 and converts an aspartic acid residue to histidine. Although this SNP was not genotyped in the present study population, it was in perfect linkage disequilibrium ($R^2=1$, $D^2=1$) in the HapMap CEPH population with the HMOX1 intronic SNP rs5995097 that was genotyped in our population. We did not detect a significant effect of this polymorphism on HMOX1 protein localization, as determined by immunohistochemistry, or on HMOX1 enzymatic activity (data not shown). Taken together, these data suggest that HMOX1 was not strongly regulated by local variants.

**Distal Regulation of HMOX1 by GPR39**

Using genome-wide association, we next tested whether distal DNA variants were associated with HMOX1 expression. Of the 718 374 SNPs that were polymorphic with minor allele frequency $>5\%$, 3 showed association with HMOX1 at $P<1e-5$ (Online Table IV). Each of these distally associated SNPs were located within gene introns, which suggests that the distally located genes regulate the expression levels of HMOX1. The 3 genes were GPR39, hypothetical protein LOC283521, and GRID1 (glutamate receptor, ionotropic, delta 1). We tested whether GPR39 and GRID1 exhibited regulation of HMOX1 basal levels using siRNA-mediated gene knockdown by 2 independent siRNA molecules (Figure 6A). GRID1 exhibited significant silencing, but no effect was observed in HMOX1 expression. However, GPR39, which was silenced to 10% to 20% of control levels, demonstrated consistent regulation of HMOX1. As predicted from the association data, GPR39 reduced the baseline expression of HMOX1 in all 3 donors tested by $\approx 50\%$ (Figure 6B). We performed linear regression for HMOX1 basal expression levels after correcting for GPR39 genotypes (rs12618338) to explore whether additional loci perturbed HMOX1 independently of GPR39. After multiple test corrections, no SNPs exhibited significant association with HMOX1 expression.
Discussion

It is clear that oxidized phospholipids significantly influence the expression of well over 1000 genes in HAECS. Although biochemical studies have revealed a number of important mechanisms underlying this response, the overall pathways involved are poorly understood. We used a systems genetics approach to model a biological network in which common, naturally occurring variations in the human population perturb responses to oxidized phospholipids. This approach integrates aspects of systems biology, in particular, network modeling, and genome-wide association. The resulting systems-level view emphasizes the diverse effects of oxidized phospholipids and provides the basis for many hypotheses relating to molecular interactions and genetic regulatory mechanisms.

The coexpression network we generated is a simplified model of the molecular interactions involved in responses to Ox-PAPC at the transcript level. In this model, genes are represented as nodes and the transcript correlations across genetic perturbations as edges. We assume that the connections identified result from shared regulatory mechanisms, which implies related functions. Of course, the model is incomplete and should be considered hypothesis generating. Our efforts in the present study were focused on the 2000 most regulated genes by Ox-PAPC to (1) define relationships and generate hypotheses likely to be most important to Ox-PAPC action, (2) simplify the network for visualization purposes, and (3) reduce computational time required for analyses. One caveat of our network is that by focusing on only 2000 transcripts, this network would not be able to capture relationships among all transcripts measured in the cell. Likewise, our network would not capture post-transcriptional relationships. To demonstrate the predictive power of the model and to elucidate molecular mechanisms of clinical interest, we explored several hypotheses using experimental perturbation and genetic association. In addition to the several known pathways reflected in this network, we observed novel interactions between GPR39 and HMOX1, between ATF4 and CHAC1, and between OKL38 and redox genes. These are discussed below.

The gene coexpression network consisted of the genes most affected by Ox-PAPC in 149 donors and defines the relationship between genes and pathways. The network, which consisted of 11 modules of highly connected genes (Figure 1; Online Figure II-XII), was constructed from transcripts quantified with and without Ox-PAPC treatment. The modules were enriched for pathways previously described for the action of Ox-PAPC, as well as others that have not been identified previously. The fact that our network was constructed with a large number of individuals, each likely contributing thousands of genetic variations that perturbed the responses to Ox-PAPC, enabled modeling of fine-grained gene relationships within and between modules. Because the precise connections are sometimes difficult to visualize in the figures, we included the top 10 most connected genes, their connection strength, and the corresponding modules for all 2000 genes input for network construction. This file is in Cytoscape network format so that the relationships may be easily visualized for genes of interest (Online Table V).

We previously reported a network analysis of Ox-PAPC–treated ECs using a small number of donors. The present network, which consists of a much larger number of individuals, enabled modeling of significantly improved relationships. For example, known relationships among UPR genes were more accurately capitated in the present study. In addition, important candidate genes not previously identified...
as UPR genes are suggested by their strong connectivity in this module. The striking enrichment for Gene Ontology categories observed in the network supports the hypothesis that coexpressed genes share functional significance (Table 1). Furthermore, known biological relationships were identified in modules of our network.

The coexpression network approach allows the formulation of 3 different kinds of hypotheses. First, it is possible to infer likely functions of genes whose functions are unknown if they are highly connected to genes with known functions. We have previously used this approach to show that CHAC1, a highly conserved protein whose function was previously unknown, plays a role in the UPR. We now identify a specific interaction (ATF4-CHAC1) based on our network analysis. Similarly, it can be hypothesized that genes in the turquoise module are likely to have a role in cell cycling, that genes in the purple module are likely to be members of the UPR, and so on. Second, the network modeling can provide information about novel mechanistic interactions between the genes. For example, the result that certain antioxidant genes, such as NRF2 and HMOX1, are tightly connected with numerous UPR genes in the blue module provides evidence of interactions between these molecules, and recent studies support this possibility. We now have implicated OKL38 in redox functions in the blue module. Importantly, we have identified OKL38 as an important regulator of HMOX1. In the present study, we showed that basal levels of HMOX1 varied considerably in our group of 149 donors; however, HMOX1 levels induced by Ox-PAPC were very similar for all donors (Figure 4A). We have now demonstrated that knockdown of OKL38 increased the Ox-PAPC-induced levels of HMOX1 by 3-fold (Online Figure XIV). This suggests that OKL38 and HMOX1 have an important combined relationship in redox regulation. Third, network modeling, when performed as a function of genetic variation, allows the formulation of hypotheses concerning genetic regulatory mechanisms. An example of this is the regulation of HMOX1 by GPR39, discussed below.

In the present report, we have focused on hypotheses relating to HMOX1. Our laboratories and those of others have implicated HMOX1 in protection against atherosclerosis, ischemia/reperfusion injury, and restenosis. In humans, a variable (GT)$_n$ dinucleotide repeat in the HMOX1 promoter has been associated with EC function, as well as clinical diseases including pulmonary disease, cardiovascular disease, renal transplantation, and idiopathic miscarriage (refer to Table 1 in Exner et al for specific citations). The present data did not replicate previous reports that protective microsatellite alleles contributed more HMOX1 mRNA. This discrepancy could be a cell-type difference between human umbilical vein ECs and HAECS. Because HMOX1 basal levels varied ≈9-fold among EC donors, whereas treated levels varied ≈2-fold (Figure 4A), we hypothesized that variable responses to Ox-PAPC for highly induced gene expression traits would predominantly be determined by baseline levels.

When we analyzed how baseline HMOX1 variation affected the network, we found that the expression of many proinflammatory genes in several modules was inversely correlated with HMOX1 expression (Figures 4B through 4E and 5A), with the notable exception of genes in the red module, which were negatively correlated with basal HMOX1 levels (Figure 5A; Table 2). Furthermore, baseline expression differences in HMOX1 significantly predicted individual responsiveness to Ox-PAPC ($R = -0.57$), such that greater HMOX1 protected against induction of endothelial transcripts by Ox-PAPC. To experimentally test the role of HMOX1 in our network, we silenced HMOX1 with siRNA and observed that proinflammatory molecules were affected (Figures 5B and 5C). A more detailed study of donors exhibiting either extremely high or low basal HMOX1 levels (as demonstrated in Figure 4A) would be an attractive strategy for identifying molecular regulators of HMOX1, but such a study would require additional samples to be collected. In addition, failure to identify HMOX1 local/cis-regulatory elements may be a power issue; however, undetected variants would likely have small effect sizes. This is supported by our recent identification of more than 1000 cis-regulatory elements of high significance in our previous study of only 96 donors. In addition, because the HAECs in the present study were grown to confluence by use of culture conditions in vitro, it is likely that the data collected in the present study would not fully recapitulate the in vivo gene expression profiles of HAECs.

To test for genetic trans-regulation of HMOX1 basal levels, we performed GWAS and identified 3 loci with suggestive probability values. The peak SNP at one of the loci occurred within the GPR39 locus on human chromosome 2. To test the possible relationship of GPR39 with HMOX1 expression, we used siRNA knockdown with 2 separate GPR39 siRNAs in HAECs from 3 different individuals. In all cases, GPR39 knockdown resulted in a substantial (approximately 40%) decrease in HMOX1 expression compared with scrambled controls (Figure 6). In contrast to GPR39, siRNA experiments for GRID1 at the chromosome 10 locus were

| Table 2. Greater HMOX1 Baseline Values Prevented Induction of Key Molecules in Ox-PAPC–Induced Pathways of Interest |
|-------|-------|-------|-------|
| Gene   | R     | P     | Pathway               |
| ATF3   | -0.40 | 5.01e-08 | UPR/ER homeostasis   |
| CEBPβ  | -0.41 | 2.74e-08 | UPR/ER homeostasis   |
| CHAC1  | -0.37 | 6.90e-07 | UPR/ER homeostasis   |
| SLC7A11| -0.36 | 1.23e-06 | UPR/ER homeostasis   |
| TRIB3  | -0.343| 1.58e-08 | Angiogenesis/UPR     |
| VEGFA  | -0.47 | 1.58e-10 | Angiogenesis/UPR     |
| IL-6   | -0.36 | 1.37e-06 | Proinflammatory cytokine |
| IL-8   | -0.28 | 2.41e-04 | Proinflammatory cytokine |
| LDLR   | -0.25 | 1.05e-03 | Sterol homeostasis   |
| E2F1   | -0.33 | 1.63e-05 | Cell cycle           |
| HMOX1  | 0.39  | 2.38e-07 | Redox                |
| TXN    | -0.34 | 6.01e-06 | Redox                |
| VCAM1  | -0.22 | 3.31e-03 | Adhesion molecule    |

ER indicates endoplasmic reticulum; ATF3, activating transcription factor 3; TRIB3, tribbles homolog 3; IL, interleukin; VCAM1, vascular cell adhesion molecule 1.
negative (data not shown). To further examine the mechanism by which GPR39 regulates HMOX1, we performed promoter-reporter studies. These indicated that GPR39 regulates HMOX1 expression by interaction with elements in the proximal 4.5-kb upstream region of HMOX1, although the precise site of interaction is unclear.

GPR39 is a G-protein–coupled receptor related to the Grelin/neurotensin receptor subfamily. Knockout mice are viable and have been characterized by increased body weight and altered intestinal function. This receptor is constitutively active in a number of cell types, and its activity has been shown to be increased by zinc in some cell types. The constitutive activity is mediated by Goq, which stimulates cyclic AMP response element–mediated transcription, and Gz12/13, which stimulates serum response element–mediated transcription (reviewed in Popovics et al53). We hypothesize that GPR39 regulates the serum response element–mediated transcription, and GPR39, should complement the larger population studies that directly examine links to clinical disease. The Ox-PAPC network serves as an additional resource for integration with conventional GWAS to elucidate gene interactions and regulatory relationships that can be tested in mechanistic studies.

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Disclosures
Wen-Pin Yang, PhD, and Paul Kayne, PhD, are employees and shareholders of Bristol-Myers Squibb. The remaining authors report no conflicts.

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Novelty and Significance

What Is Known?

- Atherosclerosis is a chronic inflammatory disease. The mechanisms underlying the initiation and propagation of inflammation remain poorly understood.
- Accumulating evidence suggests that oxidized phospholipids derived from lipoproteins entrapped in the vessel wall activate endothelial cells and are responsible in part for the initiation of inflammation.
- In vitro studies with cultured endothelial cells from different donors have shown that oxidized phospholipids influence the expression of hundreds of genes that regulate inflammation in a donor-specific manner.

What New Information Does This Article Contribute?

- We used a systems biology approach using endothelial gene expression from 149 donors and identified 11 groups of functionally related genes that played an important role in the response to oxidized phospholipids. We also identified key regulators of each group.
- Heme oxygenase 1 (HMOX1), which showed 9-fold variation in transcript levels among donors, was identified as a key regulator of one of these groups of inflammatory genes.
- A nucleotide polymorphism in G-protein–coupled receptor 39 (GPR39) was shown to be associated with the levels of HMOX1 and inflammatory genes; this was confirmed by biochemical studies.
Network for Activation of Human Endothelial Cells by Oxidized Phospholipids: A Critical Role of Heme Oxygenase 1

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SUPPLEMENTAL MATERIAL

Detailed Methods

Cell Culture

HAECs were isolated from aortic explants of 149 unique heart transplant donors in the UCLA transplant program and grown to confluence in 100mm dishes as previously described. We have previously validated our method of endothelial cell isolation on multiple cultures. We demonstrated that greater than 95% of the cells were Factor 8 positive and took up dil-acetyl LDL. In more recent studies we have demonstrated in multiple EC preparations that greater than 95% of the cells were PECAM positive (data not shown). At 90% - 100% confluence, cells were treated in 6-well dishes for 4 hours in duplicate with either media (1% serum) or with 40 ug/ml Ox-PAPC. Ox-PAPC was prepared from PAPC purchased from Avanti Polar Lipids (Alabaster, Alabama) as previously described. 47 HAEC cultures were treated with one preparation of Ox-PAPC, 44 cultures were treated with a second batch of Ox-PAPC, 5 donors were treated concurrently by batches 1 and 2, and the remaining 62 cultures were treated with another batch of Ox-PAPC. The HAECs treated by the first two batches of Ox-PAPC were the same population of cells used in our previous report of gene-by-environment interactions for expression phenotypes in human cells. The additional 62 cultures were not studied previously. This 'batch effect' was removed in downstream analysis by normalizing expression values between groups with ComBat. Among these samples were duplicate cultures from 9 donors (6 male and 3 female). Duplicate cultures were used for network construction but omitted from association testing to avoid overrepresentation of genotypes. Duplicates were used previously to demonstrate that expression profiles are conserved within HAEC donors. Cytoplasmic RNA was extracted with the RNeasy kit and treated with DNase (Qiagen, Valencia, CA). RNA concentrations were measured with the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) and quality checked with the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA).

For the PEIPC experiment, HAECs were treated at 90% confluence in 6-well dishes with either treatment media (1% serum), media containing 5 ug/ml PEIPC or 50 ug/ml Ox-PAPC. RNA was collected, quantified and checked for quality as described above. The PEIPC used for these studies was isolated from Ox-PAPC using previously described methods.

Gene Expression Profiling

RNA was prepared for hybridization to Affymetrix HT-HU133A microarrays using a standard protocol described previously. 629 arrays were used to quantify gene expression in this study. This corresponded to 158 cultures with expression data for both control and Ox-PAPC conditions. In general, duplicate arrays were averaged to determine control and Ox-PAPC expression values. However, in 12 cases, expression per condition was based on a single array. In addition, 1 culture had triplicate Ox-PAPC arrays and 4 cultures had quadruplicate Ox-PAPC arrays that were averaged to determine Ox-PAPC expression values. Before averaging, intensity values were normalized with the robust multi-array average (RMA) normalization method in R 2.5.0 using the justRMA function of the affy package of Bioconductor. An alternative CDF file was used to filter probes so that only intensity data from properly aligned probes, according to the NCBI transcriptome build 36, were used in downstream analysis as described previously. Sex was determined from heterozygous genotype calls on the X chromosome and revealed that 108 of the donors were male and 41 were female. Combat software was used to normalize expression values for sex and culture batch. Transcript expression was used to cluster the microarray samples and confirmed that no outlier arrays were used in downstream analysis. As discussed previously, we did not remove probes in probe sets containing SNPs because they did not significantly cause artificial eQTL. Array data is available in GEO accessions GSE20060 and GSE30169.
For the PEIPC experiment, Illumina HumanRef-8v2 microarrays were used according to the manufacturers protocol. Data was normalized in Bead Studio (Illumina) with the ‘Rank Invariant’ option.

**Network Construction and Visualization**

Duplicate expression measurements were averaged per condition and per donor. The 2000 most Ox-PAPC regulated genes were identified by comparing the untreated and Ox-PAPC treated values across the population with a paired t-test. The 2000 most Ox-PAPC regulated genes corresponded to those with t-test p-values <1.0e-28. 2000 genes were used so that the network would be inclusive of the most significant genes that were differentially expressed in response to Ox-PAPC, yet small enough to be visualized. Average transcript measurements for the 2000 transcripts in both conditions were used in network construction. The pair-wise adjacency matrix between genes was used to determine ‘topological overlap’ between gene pairs. Topological overlap is a function that takes into account the pair-wise correlation between genes as well as the number of common neighbors of gene pairs. The topological overlap matrix was raised to the power of 12 to emphasize the difference between transcripts with many connections and transcripts with few connections to other transcripts. The topological overlap matrix was used to identify ‘modules’ of highly co-expressed genes and the clustering dendogram of the topological overlap matrix was cut with the ‘dynamic hybrid’ method to define modules. We identified 11 modules, identified by arbitrarily assigned colors. The ‘grey module’ was not considered a true module because it contained the genes with dissimilar expression patterns. All of these analyses were performed in R using the freely accessible Weighted Gene Co-expression Network Analysis (WGCNA) software package.

Network visualization was performed in Cytoscape v2.6.3. Visualization of the whole network (Figures 1 and 5A) required filtering based on topological overlap (TO) such that only genes connected by high connection strength (TO > 0.9035) were shown in the network. This resulted in the visualization of 1090 nodes (transcripts), and 81,008 edges (connections). The network and individual modules were arranged using the ‘edge-weighted spring embedded’ option (with the exception of Figures 1B and 5A, which was arranged manually) in Cytoscape so that edge lengths corresponded to the similarity (topological overlap) between nodes. For visualization of individual modules all genes and connections were imported into Cytoscape. To reduce the complexity of the connections in the network graphs for individual gene modules, edges with the weakest connection strengths were removed until about 5% of the module genes became disconnected from the rest of the module. For a full list of genes in the network see Online Table I.

**Genotyping and Association Analysis**

Genomic DNA was isolated from HAECs using the DNeasy kit with RNase treatment (Qiagen). For HMOX1 microsatellite genotyping, a FAM-labeled PCR product was generated using the forward primer 5' GAG CCT GCA GCT TCT CAG AT 3' and the reverse primer 5' ACA GCT GAT GCC CAC TTT CT 3'. PCR conditions were as follows: 2.5mM MgCl₂, 200µM dNTPs, 0.4µM each primer, and 0.5U Platinum Taq (Invitrogen) in a final volume of 10ul. The Applied Biosystems 3730xI DNA sequencer was used to determine PCR product lengths, which ranged between 199-249 bp. This corresponded to 12-38 (GT) repeats. Microsatellite alleles were then classified as ‘short’ or ‘long’ (less than 29 repeats was considered short). Association between microsatellite alleles and HMOX1 expression was performed in PLINK using linear regression.

For SNP Genotyping, samples were randomly arrayed into three 96-well micro titer plates at 50ng/ul. Per Affymetrix Genome-wide Human SNP Array 6.0 assay protocol, 2 x 250ng of gDNA were digested by restriction enzymes NspI and StyI separately and products were ligated to respective adaptors (Affymetrix Human SNP 6.0 assay). PCR was used to amplify ligation products that were checked for size and quality by QIAxcel (Qiagen, Valencia, CA). Labeled PCR products were hybridized to the Human SNP 6.0 array. Array hybridization,
washing, and scanning were performed according to the Affymetrix recommendations. Scanned images were subjected to visual inspection and a chip quality report was generated by the Affymetrix GeneChip Operating System (command console) and the Genotyping console (Affymetrix). The image data was processed using the Affymetrix Genotyping Console using the Birdsuite algorithm to determine the specific hybridizing signal for each SNP call and copy number detection. SNPs used in association analysis were included when (i) they had minor allele frequencies > 5%, (ii) they were in Hardy-Weinberg Equilibrium (p-value > 0.001), and (iii) they had <10% missing data. This resulted in 718,374 SNPs that were used for association testing of distal-variants. The 147 unique donors that had complete expression and genotyping data (2 of the 149 unique donors lacked genotyping data) were used in association analysis. Genotype-gene expression associations (ie. eQTL) were tested using linear regression with the ‘–linear’ option in PLINK 1.4.

Since our HAEC population was derived from aortic explants of anonymous heart transplant donors, information about individuals was unknown. We sought to ascertain the population structure of the subset of 96 HAEC donors in this study that were investigated previously. As to interrogate whether gross population stratification caused spurious associations we clustered the autosomal genotypes of the HAEC donors together with members of the 3 HapMap populations. The results showed that our population was predominately Caucasian, and that removal of a few genetically different donors had little effect on global eQTLs. The results from this analysis have been extrapolated to the expanded set of 149 donors in the current study.

RT-qPCR, Western Blotting, and siRNA Knock-down

An equal amount of cDNA was made for qRT-PCR with the ABI High Capacity cDNA Reverse Transcription Kit (Foster City, CA). The Roche LightCycler 480 Master Mix and LightCycler (Roche Diagnostics, Indianapolis, IN) were used to run qRT-PCR reactions. siRNA experiments were performed as previously described. Briefly, cells were transfected at 75% confluence for 4 hours using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 40 nmole of siRNA oligos. siRNAs used in this study included HMOX1 siRNA#1 (Qiagen cat# SI00033089), HMOX1 siRNA#2 (Qiagen cat# SI00033096), GPR39 siRNA#1 (Qiagen cat# SI00430416), GPR39 siRNA#2 (Qiagen cat# SI00430423), GRID1 siRNA#1 (Qiagen cat# SI00430836), and GRID1 siRNA#2 (Qiagen cat# SI00430843). For HMOX1 knock-down, 24-48 hours post-transfection, cells were treated later with Ox-PAPC or media alone for 4 hours. RNA was then collected for RT-qPCR and HMOX1 protein was quantified with a polyclonal rabbit antibody (Stressgen/Assay Designs cat#SPA-895) using standard Western Blotting as previously described. For OKL38 knock-down, HAEC were transfected with siRNA against OKL38 or scrambled RNA. After 2 days cells were incubated for 4 hours with control medium or medium containing 40ug/ml of Ox-PAPC and RT-PCR performed on 8 genes in the blue module. The siRNA employed for the data shown caused an approximately 50% knock-down of OKL38 and an additional siRNA showed similar results. For GPR39 knock-down, HeLa cells were first transfected with two different siRNAs for GPR39 or a scrambled control. They were subsequently (after 2 days) transfected with the HMOX1 promoter constructs and luciferase assays performed after an additional day.

Heme Oxygenase Activity Assay and Protein Quantification

Heme oxygenase activity was measured by bilirubin generation as previously described. Briefly, HeLa or HAEC cells were grown to confluence in 10-cm tissue culture dishes. After treatment, cells were washed, scraped with a rubber policeman, and pelleted at 3000xg for 10 minutes. The cell pellet was suspended in MgCl₂, (2mM) phosphate (100mM) buffer (pH 7.4). The samples were sonicated on ice and then centrifuged at 1800xg at 4°C for 20 minutes. 10µL supernatant was taken to determine total protein concentration by the BCA
method (Thermo Scientific) using BSA as a standard. 250ul (about 1.5mg protein) supernatant was added to the reaction mixture containing 3mg rat liver cytosol, 20µmol/L hemin, 2mmol/L glucose 6-phosphate, 2U glucose 6-phosphate dehydrogenase, and 0.8mmol/L b-NADPH and incubated at 37°C for 1.5hr in the dark. The formed bilirubin was extracted with 1ml chloroform and the optical density spectrum (420nm-530nm) was measured by Bio-tek SYNERGYMx. The concentration of bilirubin produced in 60min/mg protein was calculated using the slope of bilirubin standard curve and the OD at 455nm and 520nm. HMOX1 protein was quantified using the HMOX1 human ELISA kit from Stressgen/Assay Designs (cat# 960-800) for Online Figures XIV-C-D. Total protein was used for the normalization of HO activity in Online Figures XIV-E-F.

**CHAC1 Luciferase Constructs**

A fragment of the human CHAC1 promoter and 5'UTR was cloned from a fosmid (Bacpac clone# G248P8704A5) and ligated upstream of the promoter-less luciferase reporter of pGL3Basic (Promega). Sequential deletions were generated using PCR, based on the identity of putative transcription factor binding sites from the TF search algorithm (http://www.cbrc.jp/research/db/TFSEARCH.html). All constructs were sequenced to verify that no errors were introduced by PCR. HEK cells were transfected with promoter constructs, along with plasmids expressing transcription factors, and CMV-Renilla normalization control in a ratio of 9:9:2, using lipofectamine 2000 (Invitrogen) according to manufacturers protocols. 24 hours after transfections, cells were harvested and assayed using the dual luciferase reporter system (Promega).

**HMOX1 Luciferase Constructs**

The HMOX1 luciferase constructs were described previously (-4.5 kb = pHOGL3/4.517 and -9.4 kb = pHOGL3/9.418).
Online Figures and Legends

Online Figure I: Ox-PAPC and PEIPC Elicit Highly Conserved Responses in HAECs. HAECs isolated from a single donor were treated with control media, 50 µg/ml Ox-PAPC, or 5 µg/ml PEIPC for 4 hours. Then, RNA was harvested and transcript profiling with microarrays was performed. The transcript-specific fold change values (log₂ scale) were calculated for Ox-PAPC and PEIPC versus control and compared with each other. In addition, PEIPC fold changes were compared with the average Ox-PAPC induced fold changes calculated across the population of 149 HAEC donors. The 2000 transcripts used to construct the co-expression network in this study are shown in panel A. Here, the average Ox-PAPC fold change across all 149 donors (x-axis) was plotted against the PEIPC fold changes ascertained in one HAEC donor. In panel B, the fold change values of Ox-PAPC and PEIPC were collected from the same HAEC donor and compared for the 2000 network transcripts. Panel C shows the correlation between the Ox-PAPC and PEIPC fold changes calculated in the same HAEC donor for the 10,376 unique transcripts detected on the Illumina microarray (detection p-value < 0.05). All correlation statistics are based on Pearson Correlation.
Online Figure II: The Blue Module. Transcripts are shaded by the degree of intra-modular connectivity, where red is the most connected, and edges signify co-expression. Transcript connections (‘edges’) are shaded according to similarity (dark blue > light blue).
Online Figure III: The Turquoise Module: Module visualization according to the same schema as Online Figure II.
Online Figure IV: The Purple Module: Module visualization according to the same schema as Online Figure II.
Online Figure V: The Yellow Module: Module visualization according to the same schema as Online Figure II.
Online Figure VI: The Green Module: Module visualization according to the same schema as Online Figure II.
Online Figure VII: The Brown Module: Module visualization according to the same schema as Online Figure II.
Online Figure VIII: The Red Module: Module visualization according to the same schema as Online Figure II.
Online Figure IX: The Pink Module: Module visualization according to the same schema as Online Figure II.
Online Figure X: The Black Module: Module visualization according to the same schema as Online Figure II.
Online Figure XI: The Magenta Module: Module visualization according to the same schema as Online Figure II.
Online Figure XII: The Greenyellow Module: Module visualization according to the same schema as Online Figure II.
Online Figure XIII: Module Similarity: Shown are the relationships between the first principal component of each module, termed the ‘Module Eigengene’ (ME). The height refers to the Euclidian distance between MEs so that the vertical distance to the nearest parent branch in the dendogram signifies module similarity.
Online Figure XIV: OKL38 Regulates Inflammatory and Anti-Inflammatory Genes: HAECs were transfected with OKL38 siRNA for 48 hrs and then treated with 40 µg/mL of Ox-PAPC for 4 hrs. Relative expression normalized to GAPDH level. sc = scramble siRNA; si = OKL38 siRNA; Ox = Ox-PAPC
Online Figure XV: NT5C2 Connections In The Green Module: For simplicity, transcripts in the green module were filtered using a more stringent connection threshold (topological overlap > 0.06) than Online Figure VI. Nodes directly connected to NT5C2 are highlighted in red.
Online Figure XVI: HMOX1 mRNA, Protein and Enzymatic Activity are Highly Correlated:
mRNA levels of HMOX1 are plotted for HeLa (A) and HAECs (B) after 4 hours of treatment with
media alone or media containing 40ug/ml Ox-PAPC. HMOX1 protein levels in HeLa (C) and
HAECs (D) were determined after additional 24-hour treatment with media by ELISA. Heme
oxygenase enzymatic activity in HeLa (E) and HAECs (F) was determined by rate of bilirubin
formation. Data from HeLa cells correspond to triplicate wells of one representative experiment.
Data from HAECs correspond to cells from two different donors. Plotted are the mean +/-
standard deviations.
Online Figure XVII: NRF2 – HMOX1 Correlations: The correlation between NRF2 and HMOX1 is shown at baseline in A, after treatment with Ox-PAPC in B, and between the baseline levels of NRF2 and the Ox-PAPC treated levels of HMOX1 in C. The R and p values are based on a Pearson correlation.
Online Figure XVIII: NRF2 is Highly Correlated to Known Redox Target Genes in HAECs:
Shown are the correlations between the Ox-PAPC treated values of NRF2 (NFE2L2) with NQO1 (A), CEBPB (B), GCLM (C), TXNRD1 (D), GSTK1(E), and B3GAT3 (F). The best-fit line through the data is shown in red and the R and p-values result from Pearson correlation.
Online Figure XIX: HMOX1 (GT)$_n$ Promoter Microsatellite Genotypes are Modestly Associated with HMOX1 Expression. In each plot, HAEC cultures are grouped by their genotype at the (GT)$_n$ polymorphism along the x-axis, where ‘SS’ indicates two short alleles, ‘SL’ is one short and one long allele and ‘LL’ indicates two long alleles. HMOX1 basal expression, expression after Ox-PAPC treatment and the calculated fold change values are plotted along the y-axes in the top, middle and bottom rows, respectively. Associations were calculated for all donors (left) and for each sex separately (males = middle column, females = right column).
Online Table Legends

**Online Table I: The Gene Co-Expression Network.** This table lists the 2000 transcripts that were included in construction of our network and their annotation, together with several network properties. These properties included, (1) Fold Change: the average change in gene expression elicited by Ox-PAPC across the population of HAECs, (2) kTotal: the number of total connections for that transcript, (3) kWithin: the number of connections within the same module, (4) kWithin.Scaled: a normalized kWithin metric where all values range between 0 and 1, and (5) module: the module membership for the gene. Note that the ‘grey module’ is not a true module. Genes are placed in the grey module if they cannot be placed into another cohesive module.

**Online Table II: Systems Genetics Analysis of Coronary Artery Disease GWAS Loci.** This table summarizes our systems genetics analysis where we intersected the loci identified in a recent meta-analysis of heart disease (Nature Genetics, 2011, 43:333-338) together with the gene expression data of HAECs of the current study. The first 4 columns relate HAEC data to the genes that reside at GWAS peaks. Specifically, whether these genes were measured in HAECs, if they were regulated by Ox-PAPC, and if contained in the co-expression network, the module they were in (columns 2-4, respectively). Columns 5-9 report HAEC results focused on the peak SNPs at the GWAS loci. Whether the peak GWAS SNP (column 5) was measured in our HAEC data is shown in column 6. Then, genes that exhibited regulation by these SNPs are shown in column 7, the class of gene expression phenotype in column 8, and the class of eQTL exhibited by the eQTL in column 9.

**Online Table III: Red Module Genes Positively Correlated With HMOX1 Basal Levels.** Listed are all red module genes and their correlations to HMOX1. These correlations were calculated using baseline HMOX1 expression and Ox-PAPC treated expression of red module genes by Pearson correlation.

**Online Table IV: Top Three distal-eQTL Associations For HMOX1 Basal Expression.** Listed are the physical positions, minor allele frequencies (MAF), association p-values, SNP types, and genes located at SNP locations for the top three associations to HMOX1 basal expression levels.

**Online Table V: The Top 10 Most Connected Genes For All Network Genes In Cytoscape Format.** The top 10 network connections as defined by topological overlap are listed in alphabetical order (according to gene symbol) for each transcript in the network and then ranked by interaction strength. The columns in this table are as follows: Unique ID = the gene symbol concatenated with the Affymetrix probe set ID used to create a unique sorting identifier, GeneSymbol_node1 = the gene symbol for the gene of interest, node1 = the Affymetrix probe set ID used in the expression measurement, module_node1 = the module containing the gene of interest, GeneSymbol_node2 = the gene symbol of the gene connected to the gene of interest, node2 = the Affymetrix probe set ID for the connected gene, module_node2 = the module containing the connected gene, interaction.type = the type of interaction between the genes (all are ‘undirected’ in this network), and the interaction.strength = the strength of the connection between the gene of interest and the other gene. Smaller interaction.strength values indicate stronger connections. This file may be imported into Cytoscape using the ‘Import Network From Table’ option.
Supplemental References


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