VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Preeclamptic Plasma Induces Transcription Modifications Involving the AP-1 Transcriptional Regulator JDP2 in Endothelial Cells

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Preeclampsia is a pregnancy disorder characterized by hypertension and proteinuria. In preeclampsia, the placenta releases factors into the maternal circulation that cause a systemic endothelial dysfunction. Herein, we investigated the effects of plasma from women with preeclamptic and normal pregnancies on the transcriptome of an immortalized human umbilical vein endothelial cell line. The cells were exposed for 24 hours to preeclamptic or normal pregnancy plasma and their transcriptome was analyzed using Agilent microarrays. A total of 116 genes were found differentially expressed: 71 were up-regulated and 45 were down-regulated. In silico analysis revealed significant consistency and identified four functional categories of genes: mitosis and cell cycle progression, anti-apoptotic, fatty acid biosynthesis, and endoplasmic reticulum stress effectors. Moreover, several genes involved in vasoregulation and endothelial homeostasis showed modified expression, including *EDN1*, *APLN*, *NOX4*, and *CBS*. Promoter analysis detected, among the up-regulated genes, a significant overrepresentation of genes containing activation protein-1 regulatory sites. This correlated with down-regulation of *JDP2*, a gene encoding a repressor of activation protein-1. The role of *JDP2* in the regulation of a subset of genes in the human umbilical vein endothelial cells was confirmed by siRNA inhibition. We characterized transcriptional changes induced by preeclamptic plasma on human umbilical vein endothelial cells, and identified, for the first time to our knowledge, *JDP2* as a regulator of a subset of genes modified by preeclamptic plasma. (*Am J Pathol* 2013, 183: 1993–2006; <http://dx.doi.org/10.1016/j.ajpath.2013.08.020>)

Preeclampsia is a pregnancy complication affecting approximately 5% to 8% of pregnancies and is capable of causing both maternal and fetal morbidity and mortality.¹ The maternal syndrome develops after 20 weeks of gestational age

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and is characterized by elevated blood pressure (>140 mm Hg/90 mm Hg), proteinuria (>300 mg/24 hours), systemic endothelial cell (EC) dysfunction, and inflammation. Defective placentation is thought to be at the root of the disease. In the developing preeclamptic placenta, the normal process of trophoblast invasion and remodeling of the uterine maternal spiral arteries is impaired. This default leads to reduced placental perfusion, oxidative stress, and inflammation, with subsequent release of placental factors and debris into the maternal circulation. These factors are supposed to cause a widespread EC activation leading to the multisystem dysfunction characteristic of preeclampsia.²

Maternal endothelial dysfunction in preeclampsia is revealed by structural modifications of the ECs of the kidney glomeruli and by functional modifications, including changes in the balance of coagulant and anticoagulant factors and increased circulating concentrations of von Willebrand factor, endothelin-1 (ET-1), soluble adhesion molecules, and cytokines. Moreover, increased levels of the anti-angiogenic factors, soluble fms-like tyrosine kinase-1 (sFLT1) and soluble endoglin, presumably released by the placenta, are found in the plasma of preeclamptic women. Therefore, endothelial dysfunction can, by itself, explain many maternal symptoms of preeclampsia. EC dysfunction will lead to hypertension due to vasoconstriction and proteinuria due to glomerular damage, and would be the cause of systemic inflammation and edema due to increased vascular permeability.^{3,4}

Targeted studies have shown that the plasma from preeclamptic women is able to elicit specific responses in human ECs *in vitro*.⁴ Thus, incubation of ECs with preeclamptic plasma increases the expression and the production of ET-1, platelet-derived growth factor, vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), cellular fibronectin, chemokines, cytokines, inducible nitric oxide (NO) synthase, NADPH oxidase 2 (NOX2), prostacyclins, NO, and reactive oxygen species (ROS).^{4–14} These studies support the idea that preeclamptic plasma contains factors able to trigger EC dysfunction.

In addition to these targeted studies, only one study, published in 2005, has investigated the effects of preeclamptic plasma on the global gene expression profiles of ECs.¹⁵ In this work, the authors used microarrays to analyze the *in vitro* gene expression profiles of human umbilical vein endothelial cells (HUVECs) and human glomerular microvascular endothelial cells after 24-hour exposition to preeclamptic or matched control plasma. However, they could not detect substantially altered gene expression by preeclamptic factors, except for a modest induction of IL-8 (approximately 1.5-fold). Therefore, they concluded that there are few endothelium-activating factors in the plasma of preeclamptic patients that can directly activate ECs. They hypothesized that the technical limitations of the 2005 microarrays were part of the reason why only mild modifications were detected, prompting us to perform a new study. Therefore, they performed a transcriptomic study on an immortalized HUVEC line exposed to plasma from normotensive or preeclamptic pregnancies. They found modifications

of gene pathways, which could be involved in long-term endothelial dysfunction. Most interestingly, several genes in these pathways present a possible regulation through the Jun family of transcription factors [ie, an enrichment in activation protein-1 (AP-1) binding sites]. Because the AP-1 negative regulator, Jun dimerization protein 2 (*JDP2*), was among the most down-regulated genes by exposure to preeclamptic plasma, they analyzed its direct implication through siRNA knockdown experiments. Our results suggest that *JDP2* is one of the factors responsible for endothelial transcriptional modifications in the preeclamptic patients.

Materials and Methods

Sample Collection, Processing, and Validation

Frozen plasma samples from women with normal or preeclamptic pregnancies were obtained from a previous epidemiological unpublished study. In all cases, informed consent was obtained. The local ethics committee of Federative Research Institute 48 (Marseilles, France) approved the use of the samples for the present project (agreement 08-012). Preeclampsia was defined using the criteria of gestational hypertension and proteinuria. Gestational hypertension was defined as new-onset systolic blood pressure >140 or diastolic pressure >90 mm Hg after 20 weeks' gestation. Proteinuria was defined as >300 mg/24-hour urine collection. Proteinuria was measured using dipstick test. All preeclamptic plasmas showed traces >300 mg/dL, corresponding to an approximate of 1 to 2 g/24 hours. Blood samples were collected in 10 mL EDTA-tripotassium Vacutainer tubes (Becton Dickinson, San Jose, CA). Within 1 hour, the tubes were centrifuged for 15 minutes at $700 \times g$ and 4°C to remove blood cells. Plasma samples were divided into aliquots and stored at –80°C. Clinical characteristics of women who provided the blood used in the study are listed in Table 1. Plasma was obtained from two groups: a group of normotensive women with uncomplicated pregnancies and a group of preterm pregnant preeclamptic women. At the moment of blood collection, the control group ($n = 10$) had an average gestational age of 36 weeks (range, 28 to 40 weeks) and had no history of hypertension, diabetes, or infection. The preeclamptic group ($n = 10$) was composed of women with preeclampsia with an average gestational age at the moment of blood sampling of 32 weeks (range, 27 to 36 weeks). On the basis of blood pressure and proteinuria values, most of the women of this group could be categorized as having severe preeclampsia. All of the procedures followed for the plasma and clinical data collection from the patients were in accordance with institutional guidelines. In addition to the clinical parameters, the plasma samples were further characterized by measuring the levels of tumor necrosis factor α (TNF- α) using Quantikine human TNF- α immunoassay (R&D Systems, Minneapolis, MN) and soluble VCAM-1 using Quantikine human soluble VCAM-1 immunoassay (R&D Systems). The levels of both TNF- α and soluble VCAM-1 were found significantly elevated in the

Table 1 Clinical Data of the Preeclamptic and Matched Pregnant Control Women Included in the Study

Data	Normal pregnancy (<i>n</i> = 10)	Preeclampsia (<i>n</i> = 10)	<i>P</i> value
Maternal age (years)	31.5 (29–34)	31 (29.5–35)	
Gestational age (weeks)	38 (32–39)	30 (30–33.5)	0.03
Race			
White	5	6	
African	4	3	
Others	1	1	
Nulliparity	3	5	
Systolic blood pressure (mm Hg)	120 (119–126)	150 (143–167.5)	1.2×10^{-5}
Diastolic blood pressure (mm Hg)	70 (70–80)	100 (94–100)	4.8×10^{-5}
Proteinuria (mg/dL)*	0	>300	

Differences between the preeclamptic and control group were analyzed using a Mann-Whitney nonparametric test. Data are given as medians (75% CIs), based on the interquartile range.

*Proteinuria was measured with a dipstick test, and levels were >300 mg/dL (approximately 1 to 2 g/24 hours).

plasma samples of preeclamptic women when compared with matched controls (Supplemental Figure S1). These analyses corroborate previous studies and indicate increased EC activation and inflammatory status in the preeclamptic women included in this study.

Endothelial Cell Cultures

An HUVEC line immortalized with the large T-antigen of SV40 and ectopic expression of the human telomerase reverse transcriptase was used in this study.¹⁶ Since the establishment of this cell line, its phenotype has been maintained over passages. Cells were routinely cultured in uncoated 75-cm² tissue culture flasks at 37°C and 5% CO₂. Culture medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mmol/L GlutaMAX (L-alanyl-L-glutamine; Invitrogen, Life Technologies, Carlsbad, CA), 5 IU/mL heparin, 100 IE/mL penicillin, and 100 mg/mL streptomycin. In addition, we used an immortalized human microvascular cell line, the human dermal microvascular endothelial cell (HMEC)-1, to confirm our results on most modified genes. The culture medium for the HMEC-1 consisted of MCDB-131 supplemented with 10% (v/v) heat-inactivated FCS, 0.2 µg/mL hydrocortisone, 10 ng/mL epidermal growth factor, 2 mmol/L GlutaMAX, 5 IU/mL heparin, 100 IE/mL penicillin, and 100 mg/mL streptomycin.

Endothelial Monolayer Permeability

Endothelial monolayer permeability was assessed by the passage of fluorescein isothiocyanate–conjugated dextran (0.1 mg/mL, 40 kDa; Invitrogen), as previously described.¹⁷ Briefly, 100,000 HUVECs were plated onto 6.5-mm Transwell collagen-coated 3-µm pore polytetrafluoroethylene membrane inserts (Costar, Corning Incorporated, New York, NY), left for 3 days to form mature monolayers, and further starved for 16 hours. After 1 hour of stimulation with 10% preeclamptic or normal plasma, each sample from the bottom chamber was read on a fluorescent plate reader (FUSION; Packard BioScience Company, Meriden, CT).

Experimental Design

In all experiments, HUVECs were grown until forming 90% confluent monolayers. Then, cells were washed twice in PBS and serum starved for 12 hours. To study the effects of preeclamptic plasma factors on endothelial gene expression, serum-starved HUVECs were grown for 24 hours in culture medium devoid of FCS but supplemented with 10% (v/v) plasma from preeclamptic patients or matched controls. To determine the responsiveness of HUVECs to proinflammatory stimulus, cells were incubated for 5 hours in complete culture medium with TNF-α. The concentrations of TNF-α were 0.1, 0.5, 1, and 5 ng/mL.

RNA Isolation

Total RNA was isolated from HUVECs with TRIzol (Invitrogen Life Technology), according to the manufacturer's instructions, and treated with DNase I to eliminate genomic DNA contamination. The quality of the RNA was analyzed using the Bioanalyzer 2100 and the RNA 6000 nano LabChip kit (Agilent Technologies, Santa Clara, CA). Only total RNA samples with an RNA Integrity Number (RIN) ≥0.8 were used.

Agilent Oligonucleotide Microarrays

For gene expression measurement, we used the Agilent whole genome microarray kit, Agilent human 8×60K (reference G4851A), interrogating a total of 27,958 Entrez gene RNAs and 7419 lincRNAs. The hybridizations were performed at the genomic and transcriptomic platform of the Cochin Institute (Paris, France), according to standardized procedures. Normalization and statistics were performed using Partek Genomic Suite (Saint Louis, MO). Raw data were pre-processed using quantile normalization, and log transformed. Unsupervised analysis on data allowed us to check undesired experimental factors and biological outliers using hierarchical clustering (Pearson's dissimilarity and average linkage) and principal component analysis, including all samples. To avoid

false-positive signals, we excluded the genes with an average expression level <100 from the analyses. In our experience, expression values from probes giving low absolute intensity signals are not reliable. To find differentially expressed genes, we applied a classic unpaired Student's *t*-test between compared groups and computed the fold-change for each gene. Then, we used these two statistics to filter and select differentially expressed genes. We selected genes with $P < 0.05$ and fold-changes <1.25. The data sets were prepared according to the guidelines of minimum information for a microarray experiment and were deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>; accession number GSE41681).

Quantitative RT-PCR

Quantitative RT-PCR was used to validate a subset of genes that showed differential expression in the HUVECs treated with preeclamptic plasma. Twelve genes were selected based on their fold differences and biological relevance. The RNA samples were reverse transcribed according to a standardized protocol. Briefly, 2 µg of total DNase-treated RNA was reverse transcribed in a volume of 25 µL at 39°C using the Superscript Reverse Transcriptase (Invitrogen) during 1 hour. Quantitative RT-PCR was performed in duplicate on eight control and nine preeclamptic samples. The amplification kit, LC480 SYBR Green Master Kit (Invitrogen), was used with 1 µM specific primers to a final volume of 10 µL. The reaction was performed in a Light Cycler Thermocycler (Roche Applied Science, Mannheim, Germany). The primers (Table 2) were designed for the coding sequences (GENBANK) of the different genes to be analyzed using the

PRIMER3 software (<http://simgene.com/Primer3>). Different couples were chosen to cover all of the previously described isoforms and aligned with basic local alignment search tool software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to avoid non-specific annealing. We used the following PCR program: 95°C for 5 minutes, followed by 35 cycles of three temperature steps (95°C for 10 seconds, 55°C for 15 seconds, and 72°C for 15 seconds). Finally, samples were submitted to a progressive temperature elevation (from 65°C to 99°C at 0.1°C/second), resulting in a fusion curve, enabling us to check the homogeneity of the PCR products. In addition, amplification products were systematically controlled by agarose gel electrophoresis. The threshold cycle number (C_T) values were collected with the LightCycler 480 software version 1.5 (Roche) using an automated second derivative method, in the exponential phase of the PCR. These C_T values were normalized by the C_T values obtained for the reporter genes, succinate dehydrogenase subunit A (*SDHA*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Functional Clustering by DAVID

Two lists of genes induced or repressed in the HUVECs cultured with preeclamptic plasma were submitted to the DAVID database.^{18,19} Briefly, DAVID clusters genes from a list according to a series of keywords common to several genes from the list. The proportion of each keyword from the gene list submitted is compared with the proportion in the whole genome, making it possible to compute a *P* value. Enrichment values are then calculated as the geometric mean of the inverse log of each *P* value.

Table 2 Primers Used in the Quantitative RT-PCR Analysis

Gene	Forward primer	Reverse primer	Amplicon size (bp)
<i>NOX4</i>	5'-TGCCATGAAGCAGGACTCTA-3'	5'-GCCACATTCTCACATTTCCTCA-3'	144
<i>BIRC5</i>	5'-CATAGAGCTGCAGGTGGAT-3'	5'-AAAACCCAGTAGGGTCCACA-3'	120
<i>CEBPB</i>	5'-CGTGTGTACACGGGACTGAC-3'	5'-AAAACAAAACAAAACATCAACAGC-3'	110
<i>ATF4</i>	5'-CCTGAAAGATTGATAGAAGAGGTC-3'	5'-TGGACACACAGCTACAGCA-3'	118
<i>VEGFA</i>	5'-GGATCAAACCTCACCAAGG-3'	5'-CCTTCTCCTCGAAGTATTTT-3'	120
<i>BCL2A1</i>	5'-GGCATCATTAAGTGGGAAG-3'	5'-TGAAATCTCCTTATAGGTATCCACA-3'	114
<i>E2F1</i>	5'-ACGCTATGAGACCTCACTGAA-3'	5'-TCCTGGGTCAACCCCTCAAG-3'	249
<i>CCNB2</i>	5'-CCGACGGTGTCCAGTGATTT-3'	5'-TGTTGTTTTTGGTGGGTGAACT-3'	180
<i>CCNA2</i>	5'-CGCTGGCGGTACTGAAGTC-3'	5'-GAGGAACGGTGACATGCTCAT-3'	120
<i>CDC20</i>	5'-GCACAGTTCGCGTTCGAGA-3'	5'-CTGGATTGCGCAGGAGTTCGG-3'	188
<i>ADAMTSL1</i>	5'-GGGGCTCCTACTCTCTGAG-3'	5'-AGTCCACATTACTGCATGTTCTG-3'	88
<i>ID3</i>	5'-CATCGACTACATTCTCGACCTG-3'	5'-TCCTTTTGTCGTTGGAGATGAC-3'	128
<i>APLN</i>	5'-GTCTCCTCCATAGATTGGTCTGC-3'	5'-GGAATCATCCAACTACAGCCAG-3'	149
<i>TGM2</i>	5'-GAGGAGCTGGTCTTAGAGAGG-3'	5'-CGGTACGACACTGAAGGTG-3'	184
<i>INSIG1</i>	5'-CCTGGCATCATCGCTGTT-3'	5'-AGAGTGACATTCTCTGGATCTG-3'	103
<i>CAMK2N1</i>	5'-GACACCAACAACCTTCTCGGC-3'	5'-TCATCTTCAATAACAACCCGCTT-3'	92
<i>EDN1</i>	5'-AACCAGGTGCGAGACCATGA-3'	5'-CCGAAGGTCTGTCCACCAATGT-3'	123
<i>GAPDH</i>	5'-AACAGCGACACCCATCCTC-3'	5'-CATACAGGAAATGAGCTTGACAA-3'	81
<i>IGFBP3</i>	5'-CTGTGGCCATGACTGAGGAAAG-3'	5'-TCCCTGAGCCTGACTTTGCC-3'	97
<i>JDP2</i>	5'-GATGCCGGAACAAGAAGAAG-3'	5'-GCTTCAGCTCCTCAATCTGG-3'	105
<i>SDHA</i>	5'-TACAAGGTGCGGATTGATGA-3'	5'-CAAAGGGCTTCTTCTGTTGC-3'	66

Functional Clustering and Biological Pathway Analysis with GENOMATIX

A list of statistically significant differentially expressed genes between HUVECs treated with normal or preeclamptic plasma was generated. This list was analyzed with several GENOMATIX tools, including GeneRanker and the Pathway analysis tool GePS (<http://www.genomatix.de/en/index.html>). The genes modified >1.25-fold were submitted as text files with the level of induction/repression. This allowed us to generate pathways with a threshold for significance in the gene clustering established at $P < 0.05$.

Pathological Association Testing

To assess the potential role of the differentially expressed genes in pathology, we used the GENOMATIX diseases Medical Subject Headings (MeSH) tool. This analysis allowed testing for an eventual association between the list of modified genes and specific keywords associated with preeclampsia, including preeclampsia, hypertension, inflammation, cardiovascular disease, and inflammation.

Promoter Analysis

The set of modified genes identified by microarray analysis was submitted to promoter analysis to identify regulatory mechanisms. The GENOMATIX Gene to Promoter tool was used to analyze the promoter regions of these genes, searching for mammalian transcription factor binding sites. A background set of promoter sequences was extracted in a similar manner using a set of unmodified genes from our microarray study. A sequence-specific overrepresentation was calculated using a t -test for comparing the content of a given promoter in a given putative transcription factor binding site. The statistical values were corrected by strict Bonferroni corrections, requesting a P value of 2×10^{-4} , given the independent test of 180 putative binding sites ($0.05/180$).

JDP2 Gene Silencing

HUVECs were plated in Dulbecco's modified Eagle's medium/GlutaMAX and 10% fetal bovine serum on day 0, and were transfected with 10 pmol of siRNA oligonucleotides targeting human *JDP2* (Qiagen, Hilden, Germany) or non-targeting controls using Lipofectamine RNAiMAX (Qiagen) on day 1, in serum-free medium. The control siRNA sequence of the sense strand was 5'-UAGCAAUGACGAAUGCGU-AdTdT-3'. The sense and antisense sequences of the duplex specific for human *JDP2* were 5'-GCCAUGAGUUGCAACCAAATT-3' and 5'-UUUGGUUGCAACUCAUGGCTT-3', respectively. After 6 hours, the medium was changed to complete medium. Total RNA was extracted after 48 hours and processed for quantitative RT-PCR. *JDP2* siRNA silencing was performed in association with human plasma treatment. In this case, after 6 hours of transfection, the

medium was changed to complete medium; and after 24 hours, cells were incubated with 10% (v/v) plasma from normal pregnancies for 24 hours and total RNA was extracted.

Statistics

Microarray analysis was analyzed using DAVID and GENOMATIX, for gene clustering.¹⁸ GENOMATIX was also used for promoter analysis. Clinical parameters of the patients were analyzed using the Mann-Whitney test. Validation performed by quantitative RT-PCR was analyzed by Student's t -test. $P < 0.05$ was considered significant.

Results

Validation of the Endothelial Cell Line Used in the Study

An immortalized HUVEC line was used to ensure the reproducibility of the results. In PE, expression of markers of proinflammatory endothelial activation and increased vascular permeability have been consistently reported. Therefore, to verify that the chosen HUVEC line was appropriate for our study, we determined its response to proinflammatory stimuli and evaluated its response to preeclamptic plasma in terms of permeability. We verified that the HUVEC line was responsive to proinflammatory stimuli by treating it with TNF- α (0.1, 0.5, 1, or 5 ng/mL) and measured the expression of several endothelial markers after a 5-hour exposition. This resulted in a dose-response up-regulation of several endothelial activation markers, including IL-1, IL-8, VCAM-I, ICAM-I, and E-selectin (Supplemental Figure S2). Also, we conducted an assay to compare the permeability of the HUVECs exposed to preeclamptic or normal pregnant plasma. This analysis showed that the preeclamptic plasma samples significantly increased the permeability of the HUVEC line, confirming that these cells display another of the characteristic response of ECs to PE (Supplemental Figure S3). Therefore, the selected HUVEC line meets basic criteria necessary to analyze the transcriptional effects of preeclamptic plasma factors on the ECs.

Microarray Analysis of the HUVECs Treated with Preeclamptic Plasma

The HUVECs were incubated for 24 hours with plasma from either preeclamptic or matched normal pregnant female plasma, and gene-expression profiles were analyzed with DNA microarrays interrogating 27,958 genes. In the preeclamptic plasma-treated HUVECs, 71 genes were significantly up-regulated and 45 were down-regulated compared with HUVECs incubated with normal pregnant plasma. Table 3 lists a selection of the differentially expressed genes with $P < 0.05$ (a full list of modified genes is given in Supplemental Table S1). We then used the DAVID version 6.7 and GENOMATIX software programs to perform functional and network analyses. This allowed us to identify gene classifiers and pathways that are

Table 3 Differentially Expressed Genes in the HUVECs Treated with Preeclamptic Plasma

Gene symbol	Gene name	Fold change
Up-regulated genes		
<i>EDN1</i>	Endothelin-1	1.88
<i>ADAMTSL1</i>	ADAMTS-like 1	1.79
<i>ID3</i>	Inhibitor of DNA binding 3	1.74
<i>NOX4</i>	NADPH oxidase 4	1.69
<i>INSIG1</i>	Insulin-induced gene 1	1.59
<i>BCL2A1</i>	BCL2-related protein A1	1.53
<i>HIST1H1D</i>	Histone cluster 1, H1 days	1.44
<i>CDC20</i>	Cell division cycle 20 homolog	1.43
<i>APLN</i>	Apelin	1.43
<i>FASN</i>	Fatty acid synthase	1.38
<i>SPARC</i>	Secreted protein, acidic, cysteine rich	1.37
<i>CCNB1</i>	Cyclin B1	1.37
<i>PSRC1</i>	Proline/serine-rich coiled-coil 1	1.36
<i>CBR3</i>	Carbonyl reductase 3	1.34
<i>KIF20A</i>	Kinesin family member 20A	1.34
<i>HMMR</i>	Hyaluronan-mediated motility receptor	1.33
<i>ICAM2</i>	Intercellular adhesion molecule 2	1.32
<i>NEK2</i>	NIMA (never in mitosis gene a)-related kinase 2	1.32
<i>HSPA5</i>	Heat shock 70-kDa protein 5	1.28
<i>BIRC5</i>	Baculoviral IAP repeat-containing 5	1.28
<i>CDCA8</i>	Cell division cycle—associated 8	1.27
<i>CCNA2</i>	Cyclin A2	1.27
<i>LOXL2</i>	Lysyl oxidase-like 2	1.27
<i>KPNA2</i>	Karyopherin α 2	1.26
<i>PTTG1</i>	Pituitary tumor-transforming 1	1.25
<i>TGFBI</i>	Transforming growth factor, β induced	1.25
Down-regulated genes		
<i>NFIL3</i>	Nuclear factor, IL-3, regulated	−1.34
<i>CITED2</i>	Cbp/p300-interacting transactivator	−1.35
<i>STC2</i>	Stanniocalcin 2	−1.44
<i>CAMK2N1</i>	Calcium/calmodulin-dependent protein kinase II inhibitor	−1.47
<i>ATF4</i>	Activating transcription factor 4	−1.50
<i>VEGFA</i>	Vascular endothelial growth factor A	−1.50
<i>TXNIP</i>	Thioredoxin-interacting protein	−1.53
<i>KLF9</i>	Kruppel-like factor 9	−1.68
<i>CBS</i>	Cystathionine- β -synthase	−1.69
<i>TSC22D3</i>	TSC22 domain family, member 3	−1.86
<i>IGFBP3</i>	Insulin-like growth factor binding protein 3	−1.93
<i>TRIB3</i>	Tribbles homolog 3	−1.96
<i>CEBPB</i>	CCAAT/enhancer binding protein, β	−2.02
<i>DDIT4</i>	DNA damage—inducible transcript 4	−2.15
<i>JDP2</i>	Jun dimerization protein 2	−2.67
<i>CHAC1</i>	Cation transport regulator homolog 1	−2.88

Partial list of genes displaying significantly modified expression ($P < 0.05$) between the HUVECs exposed to preeclamptic plasma and those exposed to plasma from normal pregnant women.

significantly enriched in HUVECs treated with preeclamptic plasma (Table 4). Expression levels of genes involved in cell cycle control and progression were increased in HUVECs treated with preeclamptic plasma, such as *PTTG1*, *CCNB1*, *CCNA2*, *TXNIP*, *PLK1*, *PSRC1*, *CDC20*, *KPNA2*, *CKAP2*, *CDCA8*, *SPHK1*, and *ID3*. Interestingly, we found that *JDP2*, a member of the AP-1 family known to suppress cell cycle progression, was down-regulated.²⁰ Another category of up-regulated genes was composed of inhibitors of the apoptotic process (*BCL2A1*, *BIRC5*, *SPHK1*, *HSPA5*, and *RTEL1*),

whereas the pro-apoptotic genes *PMAIP1*, *PHLPP1*, *DDIT4*, *IGFBP3*, and *CHAC1* were found to be down-regulated. *CHAC1* and five other down-regulated genes (*ATF4*, *CEBPB*, *TRIB3*, *XBP-1*, and *DDIT4*) are components of the unfolded protein response (UPR) pathway. This pathway constitutes a response to endoplasmic reticulum (ER) stress, and its activation is a feature of many chronic inflammatory and autoimmune diseases.²¹ *HSPA5* (alias *BiP*), which is up-regulated in our study, has been described as a master regulator of the anti-apoptotic UPR signaling network.²² Several modified genes

Table 4 Most Representative Overrepresented Biological Functions Identified by the DAVID and GENOMATIX Software in HUVECs Treated with Preeclamptic Plasma vs Normal Pregnancy Plasma

Functional category	No. of genes	Gene symbol	P value
DAVID Functional Annotation Chart			
Cell cycle	13	CKAP2 , <i>TXNIP</i> , <i>TRNP1</i> , CASC5 , CDC20 , BIRC5 , PTTG1 , SMC4 , CCNB1 , CDCA8 , SPAG5 , PLK1 , CCNA2	1.30×10^{-5}
Apoptosis	11	<i>PHLPP1</i> , CKAP2 , <i>TSC22D3</i> , <i>CHAC1</i> , BCL2A1 , <i>TRIB3</i> , BIRC5 , <i>PMAIP1</i> , <i>RTEL1</i> , <i>PHLDA1</i> , <i>DDIT4</i>	6.67×10^{-5}
Anti-apoptosis	8	<i>CEBPB</i> , <i>VEGFA</i> , <i>SPHK1</i> , BCL2A1 , BIRC5 , <i>HSPA5</i> , <i>RTEL1</i> , <i>CITED2</i>	4.70×10^{-4}
Regulation of cell migration	7	<i>VEGFA</i> , <i>EDN1</i> , SPHK1 , ITGB3 , <i>IGFBP3</i> , <i>INSR</i> , <i>CITED2</i>	9.65×10^{-4}
Negative regulation of apoptosis	9	<i>CEBPB</i> , <i>VEGFA</i> , SPHK1 , BCL2A1 , <i>RAG1</i> , BIRC5 , <i>HSPA5</i> , <i>RTEL1</i> , <i>CITED2</i>	2.61×10^{-3}
GENOMATIX Gene Ranker Analyzer			
M phase of the mitotic cell cycle	15	CDCA8 , SPAG5 , SMC4 , KIF20A , <i>EDN1</i> , <i>INSR</i> , BIRC5 , CASC5 , <i>CCNB2</i>	8.45×10^{-9}
Cell cycle	23	PTTG1 , CCNB1 , CCNA2 , <i>TXNIP</i> , PLK1 , PSRC1 , CDC20 , KPNA2 , CKAP2 , CDCA8 , SPHK1 , SPAG5 , GAS2L3 , <i>CITED2</i> , SMC4 , <i>TRNP1</i> , KIF20A , <i>EDN1</i> , ID3 , <i>INSR</i> , BIRC5 , CASC5 , <i>CCNB2</i>	2.99×10^{-6}
Fatty acid biosynthetic process	6	AGMO , <i>GGT5</i> , INSIG1 , FASN , <i>EDN1</i> , <i>TRIB3</i>	1.48×10^{-4}
Regulation of apoptosis	22	SLIT3 , <i>CHAC1</i> , <i>PHLDA1</i> , <i>PMAIP1</i> , <i>TXNIP</i> , SLIT2 , <i>IGFBP3</i> , <i>GARS</i> , HSPA5 , <i>VEGFA</i> , CKAP2 , SPHK1 , <i>PHLPP1</i> , <i>DDIT4</i> , <i>RAG1</i> , <i>CITED2</i> , <i>CLN5</i> , <i>CEBPB</i> , ID3 , <i>TRIB3</i> , BCL2A1 , BIRC5	2.38×10^{-4}
Response to hypoxia	6	CCNB1 , <i>VEGFA</i> , <i>DDIT4</i> , <i>STC2</i> , <i>CITED2</i> , <i>EDN1</i>	6.50×10^{-4}
Positive regulation of locomotion	6	ITGB3 , SLIT2 , <i>VEGFA</i> , SPHK1 , <i>EDN1</i> , <i>INSR</i>	6.50×10^{-4}
Cardiovascular system development	11	ADAMTS1 , CCNB1 , ITGB3 , SLIT2 , <i>VEGFA</i> , SPHK1 , <i>CITED2</i> , <i>ERRFI1</i> , <i>EDN1</i> , ID3 , <i>INSR</i>	8.98×10^{-4}
Anti-apoptosis	6	HSPA5 , SPHK1 , <i>CITED2</i> , <i>CEBPB</i> , BCL2A1 , BIRC5	6.41×10^{-3}
Cellular response to stress	14	PTTG1 , CCNB1 , CCNA2 , <i>ZSWIM7</i> , <i>PMAIP1</i> , PLK1 , <i>ATF4</i> , HSPA5 , <i>VEGFA</i> , INSIG1 , <i>CBS</i> , <i>STC2</i> , <i>RTEL1</i> , <i>EDN1</i>	2.00×10^{-3}

Up-regulated genes in HUVECs treated with preeclamptic plasma are boldfaced, and down-regulated genes are not.

are involved in fatty acid biosynthesis, including *INSIG1*, *FASN*, and *AGMO*, which are all targets of sterol regulatory element binding protein (SREBP). Moreover, genes involved in cardiovascular development (*ADAMTS1*, *CCNB1*, *ITGB3*, *SLIT2*, *VEGFA*, *SPHK1*, *CITED2*, *ERF11*, *EDN1*, *ID3*, and *INSR*) and endothelial vasoregulation (*EDN1*, *APLN*, *CBS*, and *NOX4*) were identified.

Pathological Association Testing

To further pursue the in silico analysis, the lists of up-regulated and down-regulated genes were submitted to the GENOMATIX Gene Ranker tool and the resulting Diseases (MeSH) table screened for terms related to preeclampsia (Table 5). This allowed the association of modified genes and disease-associated keywords. The test indicated that several MeSH annotations were significantly overrepresented in our list of modified genes, including pregnancy complications, cardiovascular diseases, vascular diseases, hypertension, pregnancy-induced inflammation, and preeclampsia.

Validation of Differential Gene Expression by Quantitative Real-Time RT-PCR

Microarray data were validated by quantitative real-time RT-PCR using specific primers for a selected subset of

genes found differentially expressed (Figure 1, A and B). The genes were selected according to their fold change and/or their putative functional relevance. Thus, *EDN1* and *JDP2* are two of the most modified genes in our study, with fold changes of 1.88 and -2.67 , respectively. Other selected genes are representative of the biological functions that appear overrepresented when performing functional analysis, using the DAVID version 6.7 or GENOMATIX (Munich, Germany) software (*CDC20* is involved in the cell cycle, and *BCL2A1*, *ID3*, and *IGFBP3* are involved in apoptosis regulation). This analysis was conducted on HUVECs incubated for 24 hours with plasma samples of 10 preeclamptic women and compared with the results obtained with 10 plasma samples from normal pregnant women. This way, we validated the modified expression of *EDN1*, *APLN*, *NOX4*, *TGM2*, *CDC20*, *BCL2A1*, *BIRC5*, *ID3*, *INSIG1*, *JDP2*, *IGFBP3*, and *VEGFA*. In addition, we tested the gene modifications in two groups of patients (severe cases: $n = 5$; average time for plasma collection, 32 weeks; and mild cases: $n = 7$; average time for plasma collection, 36 weeks). We did not find significant differences in gene expression alterations between the groups, except for *EDN1*, which was increased 1.8-fold in mild PE and 2.7-fold in severe PE ($P = 0.002$), suggesting that, except for this factor, the modifications observed are general for preeclampsia (Supplemental Figure S4).

Table 5 GENOMATIX Disease MeSH Terms Associated with the List of Modified Genes in HUVECs Exposed to Preeclamptic Plasma

MeSH term	P value	Observed	Expected	Total	List of observed genes
Neovascularization, pathologic	2.47×10^{-9}	38	14.09	2238	PTTG1, ADAMTS1, CCNB1, CCNA2, PMAIP1, ITGB3, TXNIP, IGFBP3, FAP, PLK1, RNASE1, ATF4, HSPA5, AKAP12, VEGFA, FST, NRGN, SPHK1, DDIT4, SPARC, STC2, RAG1, CITED2, NOX4, HMMR, APLN, FASN, EDN1, TGFBI, ICAM2, AKR1C3, CEBPB, ID3, TRIB3, MTHFD2, INSR, BCL2A1, BIRC5
Pregnancy complications	1.24×10^{-7}	48	23.9	3795	PTTG1, CCNB1, CCNA2, PMAIP1, ITGB3, TXNIP, HBZ, SLIT2, IGFBP3, PLK1, RNASE1, ATF4, CDC20, HSPA5, AKAP12, VEGFA, DCBLD2, INSIG1, MAOA, KPNA2, FST, NRGN, CBS, SPHK1, CLIC3, HMGCS1, SPARC, STC2, KLF9, RAG1, CITED2, RPGR, NOX4, KIF20A, RTE1, APLN, FASN, EDN1, ICAM2, AKR1C3, CEBPB, TROAP, ID3, TRIB3, MTHFD2, INSR, BCL2A1, BIRC5
Cardiovascular diseases	3.72×10^{-7}	70	45.05	7153	TRPM2, TSC22D3, PTTG1, TMEM195, SLIT3, ADAMTS6, ADAMTS1, CCNB1, GGT5, PHLDA1, CCNA2, PMAIP1, ITGB3, TXNIP, SLIT2, IGFBP3, FAP, PLK1, RNASE1, GARS, ATF4, PSRC1, CDC20, HSPA5, AKAP12, LOXL2, VEGFA, DCBLD2, HIST1H1D, INSIG1, PPYR1, TUBA8, MAOA, FST, NRGN, CKAP2, JDP2, CBS, SPHK1, PHLPP1, DDIT4, HMGCS1, SPARC, STC2, MXD3, RAG1, CITED2, ERFF1, RPGR, NOX4, HMMR, PDE4DIP, KIF20A, APLN, FASN, EDN1, MARS, TGFBI, ICAM2, AKR1C3, CEBPB, ID3, TRIB3, MTHFD2, INSR, BCL2A1, BIRC5, NFIL3, CBR3, CASC5
Brain ischemia	1.72×10^{-6}	32	59	2159	ADAMTS1, CCNB1, PHLDA1, CCNA2, PMAIP1, ITGB3, TXNIP, IGFBP3, RNASE1, ATF4, HSPA5, VEGFA, INSIG1, MAOA, FST, NRGN, CBS, SPHK1, DDIT4, SPARC, STC2, RAG1, CITED2, NOX4, HMMR, APLN, EDN1, CEBPB, TRIB3, INSR, BCL2A1, BIRC5
Vascular diseases	2.06×10^{-6}	62	38.76	6155	TRPM2, TSC22D3, PTTG1, TMEM195, ADAMTS6, ADAMTS1, CCNB1, PHLDA1, CCNA2, PMAIP1, ITGB3, TXNIP, IGFBP3, FAP, PLK1, RNASE1, GARS, ATF4, PSRC1, CDC20, HSPA5, AKAP12, LOXL2, VEGFA, DCBLD2, HIST1H1D, INSIG1, MAOA, FST, NRGN, CKAP2, JDP2, CBS, SPHK1, PHLPP1, DDIT4, HMGCS1, SPARC, STC2, MXD3, RAG1, CITED2, ERFF1, RPGR, NOX4, HMMR, KIF20A, APLN, FASN, EDN1, MARS, TGFBI, ICAM2, AKR1C3, CEBPB, ID3, TRIB3, INSR, BCL2A1, BIRC5, NFIL3, CASC5
Placental insufficiency	5.32×10^{-6}	9	1.27	203	CCNB1, PMAIP1, ITGB3, IGFBP3, VEGFA, CBS, NOX4, EDN1, INSR
Ischemia	4.21×10^{-5}	23	9.4	1494	PTTG1, ADAMTS1, CCNA2, PMAIP1, ITGB3, IGFBP3, ATF4, HSPA5, VEGFA, MAOA, FST, CBS, SPHK1, RAG1, NOX4, APLN, FASN, EDN1, ICAM2, TRIB3, INSR, BCL2A1, BIRC5
Inflammation	4.08×10^{-4}	43	27.05	1405	TRPM2, TSC22D3, ADAMTS1, CCNB1, PMAIP1, ITGB3, TXNIP, SLIT2, IGFBP3, RNASE1, GARS, ATF4, CDC20, HSPA5, LOXL2, VEGFA, LXN, INSIG1, MAOA, FST, NRGN, CBS, SPHK1, DDIT4, SPARC, RAG1, CITED2, ERFF1, NOX4, HMMR, APLN, FASN, EDN1, MARS, TGFBI, ICAM2, CEBPB, ID3, TRIB3, INSR, BCL2A1, BIRC5, CASC5
Hypertension, pregnancy induced	2.21×10^{-3}	15	6.58	1045	ITGB3, IGFBP3, HSPA5, VEGFA, MAOA, FST, CBS, CLIC3, STC2, APLN, EDN1, ICAM2, AKR1C3, INSR, BIRC5
Preeclampsia	4.05×10^{-3}	14	6.32	1004	ITGB3, IGFBP3, HSPA5, VEGFA, MAOA, FST, CBS, CLIC3, STC2, EDN1, ICAM2, AKR1C3, INSR, BIRC5
Kidney diseases, cystic	5.20×10^{-3}	12	5.14	817	CCNB1, CCNA2, HBZ, IGFBP3, PLK1, VEGFA, NRGN, SPARC, EDN1, INSR, PGP, BCL2A1
Embolism and thrombosis	2.76×10^{-3}	18	8.84	1405	ADAMTS1, ITGB3, IGFBP3, HSPA5, VEGFA, DCBLD2, CKAP2, CBS, SPARC, RAG1, NOX4, APLN, EDN1, MARS, TGFBI, ICAM2, CEBPB, INSR

Up-regulated genes in HUVECs treated with preeclamptic plasma are boldfaced, and down-regulated genes are not.

Promoter Analysis

To identify master genes of the preeclamptic plasma-induced transcriptomic modifications, we proceeded to an analysis of

the predicted binding sites for transcription factors present in the promoters of the differentially expressed genes. The occurrence of binding sites for transcription factors was obtained from the MatInspector Genomatix software for the

Figure 1 Validation of differentially expressed genes in HUVECs exposed to preeclamptic plasma. Selected differentially expressed genes in HUVECs exposed to preeclamptic plasma were analyzed by quantitative real-time RT-PCR to validate the results obtained with the DNA-microarray experiment (**A** and **B**). Relative gene expression values were adjusted to the mean value of the control group (Co; untreated cells) set at 1 for each gene. Gray bars, control group; black bars, preeclampsia. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$ using the Student's *t*-test.

promoters of 44 most up-regulated, 9 most down-regulated, and a sample of 57 nonmodified genes. This allowed the identification of overrepresentation in the modified gene set of putative transcription factor binding sites for AP-1 ($P < 0.0024$; $P < 0.0005$ when only the induced genes were

compared with the unmodified set). Interestingly, *JDP2*, one of the most down-regulated (-2.67) genes in the HUVECs exposed to preeclamptic plasma, encodes the Jun dimerization protein 2, a member of the AP-1 family that functions as a repressor of the AP-1-dependent transcription.

Role of JDP2 in the Transcriptomic Response of HUVECs to Preeclamptic Plasma

To focus on the role of *JDP2* in the transcriptomic activation of AP-1-regulated genes induced by the preeclamptic plasma, HUVECs were treated with an siRNA targeting *JDP2* (siJDP2). The cells were transfected with either the siJDP2 or a control (scrambled) siRNA and cultured for 24 hours in a medium supplemented with 10% normal plasma from pregnant women. Subsequently, quantitative RT-PCR was used to analyze the expression of 18 genes, containing ($n = 8$) or lacking ($n = 10$) AP-1 regulatory sites in their proximal promoters. As shown in [Figure 2](#), siJDP2 reduced the levels of *JDP2* mRNA in the HUVECs by 75%. *JDP2* knockdown resulted in significantly increased expression levels of three genes (*CAMK2N1*, *BCL2A1*, and *CEBPB*). Of these genes, only two (*BCL2A1* and *CEBPB*) are known to contain AP-1 regulatory sites in their promoter. The siRNA *JDP2* silencing resulted in significant down-regulation of 11 genes, 6 of them containing AP-1 sites (*EDN1*, *TGM2*, *CDC20*, *ID3*, *E2F1*, and *CCNA2*). Finally, the siRNA *JDP2* had no effect on the expression of nine genes. Of these genes, three contained AP-1 sites in their promoters (*NOX4*, *INSIG1*, and *ATF4*). The same experiment was repeated using another endothelial cell line, the HMEC-1, to determine whether the effects of *JDP2* down-regulation were restricted to the HUVEC line. This analysis showed that, in general, the genes modified by *JDP2* siRNA in the HUVECS are overall modified with a similar expression profile in HMEC-1, albeit with a decreased intensity, some of them being unmodified, such as *EDN1* ([Supplemental Figure S5](#)).

Figure 2 Effect of *JDP2* knockdown on the expression of preeclamptic plasma deregulated genes. To inhibit *JDP2* expression, HUVECs were transfected with 30 pmol of siRNA specific for *JDP2* (siJDP2). The same amount of nonspecific double-stranded RNA was used as a negative control (siCO). Two days after the transfection, the cells were harvested and total RNA was subjected to quantitative RT-PCR using primers specific for *JDP2* and for several genes found to be modified by the preeclamptic plasma. Levels of expression were normalized to that of GAPDH and SDHA. Data are shown as relative gene expression values adjusted to the mean value of the control group (siCO), which was set at 1 for each gene. Error bars represent the SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ using the student's *t*-test.

Figure 3 Effects of TNF- α inhibition on the expression of a selection of genes modified by preeclamptic plasma. HUVECs were cultured for 24 hours in quadruplicate, in the presence of preeclamptic plasma with or without 10 μ g/mL of the TNF- α inhibitor, etanercept. Subsequently, the expression of several genes found to be modified by the preeclamptic plasma in the previous experiments was analyzed using quantitative RT-PCR. Expression ratios, with or without etanercept, are shown for the 12 genes analyzed (a ratio of 1 meaning no effect of TNF- α inhibition). Inhibition of TNF- α signaling had a significant effect only on NOX4 expression. * P = 0.02. A trend was, however, observed for BCL2A1 (** P = 0.07) and VEGFA (*** P = 0.08). Error bars represent the SDs.

TNF- α Mediates Some Effects of Preeclamptic Plasma on Endothelial Cells but Does Not Regulate *JDP2* Expression

TNF- α is known to induce proinflammatory activation in ECs, and increased circulating levels have been reported in preeclampsia. Indeed, the measurements of cytokine levels in the plasma samples of the women used in our study indicates that the TNF- α levels are significantly higher in the plasma samples from preeclamptic women (1.5 pg/mL) than in the controls (0.5 pg/mL) (Supplemental Figure S1). Thus, we investigated whether some of the gene expression modifications induced by the preeclamptic plasma were caused by the increased levels of TNF- α , and if they were related to *JDP2* down-regulation. To this end, HUVECs were treated for 24 hours with preeclamptic plasma in the presence or absence of etanercept (an inhibitor of TNF- α). Subsequently, the expression of some genes (*JDP2*, *EDN1*, *APLN*, *NOX4*, *BIRC5*, *ID3*, *IGFBP3*, *TGM2*, *BCL2A1*, *INSIG1*, and *VEGFA*), which were found significantly modified in the microarray analysis, were analyzed by quantitative PCR. We found that TNF- α inhibition did not block the down-regulation of *JDP2* induced by the preeclamptic plasma, suggesting that this effect is mediated by other factors. Among the 12 genes tested, we observed that etanercept was able to bring the expression levels of one of them, *NOX4* (not modulated by *JDP2*), back to the level of expression in HUVECs treated with control plasma; it was induced more than twofold by preeclamptic plasma alone (Figure 3), suggesting that *NOX4* regulation passes through TNF- α activation in a *JDP2*-independent pathway. Reciprocally, it suggests that increased TNF- α levels account only for a subset of genes modified in preeclamptic plasma-treated ECs.

Discussion

In this study, we have analyzed the effects of plasma from women with either preeclamptic or normal pregnancies on

the global gene expression profile of immortalized HUVECs that were exposed for 24 hours to preeclamptic or normal pregnancy plasma. We detected 116 genes with significantly modified expression in cells exposed to plasma from preeclamptic versus normal pregnancies. Some of these genes are most relevant to EC pathophysiological characteristics, and involved in vasoregulatory functions, mitosis and cell cycle progression, apoptosis regulation, fatty acid biosynthesis, and endoplasmic reticulum stress-response. The role of these genes in the context of preeclampsia and EC physiological characteristics is discussed later.

Genes Involved in Vasoregulatory Functions, Free Radical Production, and Angiogenesis Are Altered by the Preeclamptic Plasma

Several genes identified as modified by the preeclamptic plasma are known to directly modify the properties of the endothelial vessels and, thus, are able to promote hypertension. Among those, *EDN1*, *APLN*, and *CBS* were significantly modified. *EDN1*, encoding ET-1, was the most up-regulated gene in the HUVECs treated with preeclamptic plasma. ET-1, mainly produced by ECs, possesses potent vasoactive activity. It has been implicated in the pathophysiological characteristics of many cardiovascular diseases.^{23,24} Circulating levels of ET-1 are increased in the blood of preeclamptic women, and several factors released by the preeclamptic placenta (including sFlt1, inflammatory cytokines, and agonistic angiotensin II type-1 receptor autoantibodies) are known to induce ET-1 expression. These factors induce hypertension in animal models through the production of ET-1, strengthening the possibility that ET-1 may be a mediator in the genesis of PE syndrome secondary to anti-angiogenic factors released by the placenta.

Another up-regulated gene in our experiment is *APLN*, encoding apelin, a biologically active peptide present in several isoforms that are agonists for the orphan G-coupled receptor, APJ. In the cardiovascular system, apelin is present in both the endothelium and vascular smooth muscle

cells. Acting on the endothelium, it releases NO, which mediates vasodilation, whereas acting directly on vascular smooth muscle cells, it causes vasoconstriction.²⁵ A possible role of apelin in hypertension, initial stages of heart failure, and ischemic heart disease has been suggested.²⁶ Apelin levels have previously been found increased in the placenta and plasma of preeclamptic women.^{27,28}

Among genes involved in vasoregulatory functions and down-regulated in our experiment, we found *CBS*. This gene encodes the cystathionine- β -synthase, an enzyme catalyzing the first and rate-limiting step of the trans-sulfuration pathway resulting in the conversion of homocysteine to the cysteine precursor, cystathionine. *CBS* can also catalyze the condensation of cysteine with homocysteine to form cystathionine and hydrogen sulfide (H_2S). H_2S is known to induce vasorelaxation by opening ATP-sensitive K channels in smooth muscle cells and up-regulating *VEGF*. Furthermore, H_2S has an antioxidant activity by directly scavenging NO and ROS.²⁹ Endothelial dysfunction has been observed in *Cbs*-deficient mice, both homozygotes or heterozygotes.^{30,31} Isolated vessels from these animals display reduced dilatory response to endothelium-dependent vasodilators (bradykinin, acetylcholine, and β -methacholine). *CBS* down-regulation has also been reported in the placental endothelium of early-onset preeclampsias.³² Altogether, these data show that *CBS* could play a significant role in vasoregulation, and its involvement in the endothelial dysfunction of preeclampsia seems plausible. In sum, the up-regulation of *EDN1* and *APLN* and the down-regulation of *CBS* in our experiment are consistent with their role in vasoregulatory functions and their known implication in the preeclamptic syndrome.

In relation to free radical production, and their deleterious effects in the context of preeclampsia, we found significant up-regulation of *NOX4*. This gene encodes an NADPH oxidase isoform. In ECs, *NOX4* has been identified as a major source of ROS.^{33,34} Excessive ROS production has been convincingly implicated in vascular pathological conditions. Some studies indicate that *NOX4* up-regulation activates ECs, arrests proliferation, and causes apoptotic and necrotic death.³⁵ However, *NOX4* overexpression promoted EC proliferation and inhibited apoptosis, whereas *NOX4* silencing or expression of a dominant-negative form of *NOX4* impaired EC proliferation.³⁶ Further studies are needed to resolve these conflicting observations. Notwithstanding, it is likely that increased expression of *NOX4* in ECs would lead to increased ROS production. ROS will activate various reduction-oxidation-sensitive kinases, such as Akt, Src, and mitogen-activated protein kinase, as well as transcription factors, including NF- κ B, AP-1, p53, Ets, and hypoxia-inducible factor-1, thereby increasing reduction-oxidation-sensitive gene expression, regulating growth, apoptosis regulation, migration, angiogenesis, permeability, and inflammation.³⁴ A recent study has demonstrated that, through ROS production, *NOX4* mediates the expression of plasminogen activator inhibitor-1 (*PAI-1*) via the p38

mitogen-activated protein kinase pathway in cultured human ECs.³⁷ In this way, *NOX4* could contribute to the procoagulant status observed in preeclampsia. By using entanercept, we show herein that *NOX4* transcriptional induction in ECs exposed to preeclamptic plasma is TNF- α dependent, whereas TNF- α levels are increased in preeclamptic plasma. Our results suggest, therefore, that inhibition of *NOX4* transcription or activity could be a target for therapeutic approaches.

Another way of modifying the access of oxygen to the tissue, in addition to vasoconstriction/vasodilation, is the building of the vascular network. To this respect, we found herein that the gene encoding the vascular endothelial growth factor A (VEGFA), an important actor of endothelial proliferation, migration, permeability, survival, and vasodilation, is reduced in the HUVECs treated with preeclamptic plasma. In PE, the amount of bioavailable VEGF is decreased because the ischemic placenta releases sFlt1 into the maternal circulation. sFlt1 binds free VEGF and makes it unavailable for signaling via membrane-bound receptors. Endothelial-derived VEGFA could play an essential role in maintaining endothelial homeostasis by regulating key vascular proteins, such as vascular endothelial growth factor receptor-2, tyrosine kinase with immunoglobulin and EGF homology domains-2, and vascular endothelial cadherin.^{38,39} Our results suggest that, in preeclampsia, the ECs might be confronted with a double lack of bioavailable VEGFA through the loss of paracrine and autocrine VEGFA signaling.

Cell Proliferation versus Apoptosis in ECs Exposed to Preeclamptic Plasma

Several up-regulated genes encode proteins that drive cell proliferation, suggesting that the preeclamptic plasma could be enriched in growth factors compared with normal pregnant plasma. This is consistent with other studies that have reported that preeclamptic plasma stimulates EC proliferation.⁴⁰ Several factors present in the preeclamptic plasma at increased concentrations could be responsible for this effect: VEGF, ET-1, and oxidized low-density lipoprotein. In addition, increased ROS production subsequent to *NOX4* up-regulation could also increase the expression of genes involved in EC growth and cell cycle progression, as previously mentioned. Consistently, a recent study comparing the transcriptome of peripheral blood mononuclear cells from preeclamptic and normal pregnant women found that many of the up-regulated genes in the preeclamptic peripheral blood mononuclear cells are involved in mitosis and cell cycle progression.⁴¹ Strikingly, several genes up-regulated in this study are also found up-regulated in our experiment, including *CCNB1*, *CCNB2*, *CDC20*, *CDCA8*, *NEK2*, and *CASC5*. Although proliferation is favored, anti-apoptotic genes were consistently up-regulated in the HUVECs treated with preeclamptic plasma, such as *BCL2A1*, *BIRC5*, and *ID3*. *BCL2A1* encodes a member of the BCL-2 protein family acting as an anti-apoptotic regulator^{42,43}

capable of blocking caspase activation and whose expression has already been found up-regulated in the placenta of severe preeclampsias.⁴⁴ *BIRC5* encodes the baculoviral IAP repeat-containing protein (also designated as survivin) and functions as a pivotal regulator of programmed cell death and mitosis. It interacts with the products of several genes found up-regulated in our study, such as *CDCA8*, *CDC20*, *CCNB1*, and *CCNB2*. *ID3* is a member of the basic helix-loop-helix family implicated in the pathobiological characteristics of vascular diseases of rodents, pigs, and humans.^{45–47} The increase in the expression of anti-apoptotic genes (such as *BCL2A1*, *BIRC5*, and *ID3*) and the concomitant down-regulation of pro-apoptotic genes (such as *IGFBP3*), the expression of which is modified in the placenta and in the plasma of preeclamptic women,⁴⁸ *DDIT3* or *CHAC1*) could be an adaptive response to plasma factors (inflammatory cytokines and anti-angiogenic factors) that might trigger stress-challenging cell survival. Regulation of the apoptosis pathway by the preeclamptic plasma is also substantiated by the down-regulation of the pro-apoptotic branch of the UPR pathway. Indeed, our transcriptome analysis shows down-regulation of the genes *ATF4*, *CEBPB*, *CHAC1*, *TRIB3*, *XBP-1*, and *DDIT4* on exposure of the HUVECs to preeclamptic plasma. The UPR is activated in response to an overload of misfolded proteins in the ER. Stress sensors in the ER, such as ATF6, initiate signals on the cytosolic face of the ER to reduce protein synthesis, promote protein folding, and increase the degradation of misfolded proteins. Failure of this response to alleviate protein misfolding stress leads to late expression of proteins, such as DDIT3 (also designated as CHOP-10) or CHAC1, culminating in cell death.⁴⁹ ER stress and UPR pathways have strong links to major inflammatory and stress signaling networks, including the activation of the JNK-AP1 and NF- κ B–IKK pathways, as well as production of ROS and NO.^{50–52} In addition, the chaperone, HSPA5(BiP), which was up-regulated in the present study, has been proposed as a master regulator of the anti-apoptotic UPR signaling network.²² Increased expression of *HSPA5(BiP)* is induced by several factors, including elevated levels of ROS and intracellular homocysteine.^{53,54} Homocysteine-induced ER stress results in the induction of SREBP-dependent genes involved in the biosynthesis and uptake of cholesterol and triglycerides and leads to the accumulation of cholesterol in cultured HUVECs.⁵⁵ We show herein that HUVECs exposed to the preeclamptic plasma up-regulate insulin-induced gene 1(*INSIG1*), a key component of the sterol regulatory element binding protein (SREBP-mediated regulation of cholesterol biosynthesis).⁵⁶ *INSIG1* binds to the sterol-sensing domain of SREBP cleavage-activating protein and retains the sterol-sensing domain of SREBP cleavage-activating protein/SREBP complex in the ER, preventing the translocation of the N-terminal domain of SREBP into the nucleus. This blocks SREBP from acting as a transcription factor for the genes involved in lipids and cholesterol biosynthesis (including *INSIG1*). A series of studies have demonstrated that EC inflammatory activation

by oxidized phospholipids depletes cholesterol and activates SREBP nuclear translocation, with a concomitant increase in *INSIG1* mRNA levels.⁵⁷ Increased circulating levels of triglycerides, low-density lipoproteins, and lipid peroxides have been found in the plasma of preeclamptic women.^{58,59} In addition to *INSIG1*, several other genes involved in fatty acid biosynthesis were found to be up-regulated in our study, including *HMGCS1* and *FASN*, which are both targets of SREBP.

In sum, the modifications detected in HUVECs treated with preeclamptic plasma are coherent with what is known about endothelial dysfunction in preeclampsia. Deregulation of genes encoding vasoregulatory functions (*EDN1*, *APLN*, and *CBS*) can be linked to hypertension resulting from vasoconstriction. The concomitant up-regulation of genes encoding anti-apoptotic effectors and down-regulation of pro-apoptotic factors (including those involved in ER stress control) could be an adaptive response to plasma factors (ie, inflammatory cytokines and anti-angiogenic factors), which might trigger stress-challenging cell survival.

Is JDP2 a Band Master of Endothelial Stress in Preeclampsia?

One of the most striking findings of our analysis was the discovery that many of the modified genes contain AP-1 binding sites in their promoters. This correlates with the fact that *JDP2* was one of the most down-regulated genes in the HUVECs treated with preeclamptic plasma, thus suggesting, for the first time to our knowledge, an involvement of this regulation cascade in endothelial defects in preeclampsia. *JDP2* encodes JDP2, a member of the AP-1 family implicated in many cellular processes, including carcinogenesis, cell differentiation, and cell proliferation. JDP2 is an 18.7-kDa protein able to homodimerize, or heterodimerize, with other AP-1 members, such as c-Jun, JunB, JunD, DDIT-3 (CHOP-10), ATF2, and a member of the C/EBP family, C/EBP γ . JDP2 acts as a repressor of AP-1, cAMP-response element, and TPA-responsive element-dependent transcription.^{60,61} Although JDP2 acts generally as a repressor, it has been reported that, depending on the context and cell type, it can alternatively act as a transcriptional activator.^{62,63}

In the present experiment, it was tempting to speculate that the up-regulation of several genes could result, at least in part, from the down-regulation of *JDP2*. We tested this hypothesis by inhibiting *JDP2* expression in the HUVECs using a *JDP2* siRNA, followed by the analysis of the expression of a subset of genes containing AP-1 sites in their promoters and up-regulated by the exposure to preeclamptic plasma. *JDP2* silencing resulted in the up-regulation of one gene (*BCL2A1*) of 16 genes found up-regulated by preeclamptic plasma (and containing AP-1 sites in their promoter regions). *JDP2* siRNA inhibition also leads to the down-regulation of *IGFBP3* and *VEGFA*, two genes that are down-regulated in the HUVECs exposed to preeclamptic plasma. Thus, down-regulation of *JDP2* by

the preeclamptic plasma could directly explain at least the transcriptional modification of *BCL2A1*, *IGFBP3*, and *VEGFA*. Concerning the other genes, it is probable that the up-regulation of these genes requires the down-regulation of *JDP2* and the activation or down-regulation of other cofactors. Likely, the activity of such cofactors could be dependent on signals that can be provided by the preeclamptic plasma but not by the normal pregnancy plasma that could adapt the cells by countering the effect of *JDP2* repression. Nevertheless, the fact that the single inhibition of *JDP2* modifies their expression argues in favor of a role of *JDP2* in their regulation, at least in the context of preeclampsia.

In summary, the significant response of HUVECs after 24 hours of exposition to preeclamptic plasma may help to understand the effects of preeclampsia on ECs, and allow the identification of the starting point of putative cascades of gene deregulations, which might be the source of long-term endothelial disorders in the patients. The data generated by our study set a novel base for future studies aiming to elucidate the effects of preeclampsia circulating factors on the physiological characteristics of ECs.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.08.020>.

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