Supplement Material

Results and Discussion

A total of 42 HUVECs were isolated from 42 single pregnancies (~40% c-sections). The characteristics of the subjects are shown in Table II. All pregnancies were from normotensive, non-obese healthy mothers. There were significant differences between Dex-sensitive HUVECs and Dex-resistant HUVECs in maternal body mass index and systolic blood pressure (Table II), with Dex-resistant HUVECs originating from mothers having a lower BMI and systolic blood pressure compared to Dex-sensitive HUVECs. However, there were no other differences, including newborn sex, race (as self-determined by both parents), birth weight, or umbilical vein cord plasma levels of cortisol (Table II).

We noted that the Dex-sensitivity differences observed in our HUVEC population were reproduced under various conditions, such as, different types of media, types of assays and cell passage number. For instance, we found that Dex treatment upregulated the expression of Factor VIII, vonWillebrand factor and ICAM1; while downregulating VEGFA in Dex-sensitive HUVECs in Dex-sensitive cells, but not in Dex-resistant cells (Table III). To determine the ability of Dex to stimulate GRE-mediated transactivation, we performed luciferase assays using a minimal-GRE-luciferase vector. We found that Dex induced a dose-dependent transactivation of the reporter gene in all HUVECs, but the effect was stronger in Dex-sensitive HUVECs compared to Dex-resistant HUVECs (Table IV). Finally, we analyzed the ability of Dex to increase the pro-coagulant activity of HUVECs. We found that Dex increase the pro-coagulant activity of HUVECs in a dose dependent manner in Dex-sensitive, but not in Dex-resistant,

HUVECs (Table V). Altogether, we confirmed that there are significant inter-individual differences to Dex-sensitivity in vitro, and that these differences are not due to environmental or stochastic factors.

Next, we analyzed the levels of GR isoform transcripts in our HUVEC population using quantitative real-time PCR. We did not find significant differences between our Dex-sensitive and Dex-resistant HUVEC groups in terms of GRα and GRβ mRNA levels in any condition tested (Table VI). Dex-sensitive HUVECs responded to Dex in a dose-dependent upregulation of GRα transcript levels, but the levels of GRα in Dex-treated HUVECs was not significantly different between Dex-sensitive and Dex-resistant HUVECs at any given treatment dose of Dex (Table VI). The transcriptional regulation of GRα in HUVECs has been examined and our data will be presented and discussed elsewhere.

Studies on the GR protein levels and the GR-chaperone system revealed that Dex-resistant HUVECs have a higher GR protein turnover by the proteosomal system. These events are chaperoned by various isoforms of BAG1, in particular BAG1L and BAG1S. However, there were no significant differences in the interactions of GR with other chaperones such as HSP90, HSP70, FKBP51 and FKBP52 (Figure II-A). Neither were there differences in the levels of cytosolic and nuclear chaperones HOP, HIP, HSP90, HSP70, FKBP52, FKBP51 and PTGES between Dex-sensitive and Dex-resistant (Figure II-B). Furthermore, differential BAG1 expression was evident only in confluent HUVECs (which were used throughout this study), with no significant differences in GR and BAG1 isoform expression between Dex-sensitive and Dex-

resistant HUVECs in proliferating cells (Figure II-C). The significance of these results are discussed in the original manuscript.

To confirm the role of BAG1 on GR protein expression and function, we performed overexpression and silencing assays, which are shown in the main manuscript. To account for possible off-target effects, we also investigated the effect of overexpression and silencing of BAG1 on GR chaperone expression. Figure III-A shows that overexpression of BAG1 isoforms did not induce overexpression of other GR chaperones. However, we observed that FKBP51 was significantly upregulated by Dex in Dex-sensitive cells, and BAG1 isoforms blocked this effect (Figures III-A and III-B). FKBP51, similar to PAI-1, contains GRE sites in its promoter, and is therefore responsive to Dex-mediated transcriptional upregulation (1).

Similar to our overexpression studies, silencing of BAG1 isoforms did not result in off-target silencing of other GR chaperones (Figure III-C). However, we did observe that BAG1 silencing led to significant increases in HSP70 expression (Figures III-C and III-D). Finally, BAG1 silencing also restored Dex-sensitivity in Dex-resistant cells as shown by upregulation of FKBP51 expression (Figures III-C and III-E).

We then investigated the role of BAG1 on GR expression in other human vascular cells, and our results are shown in the main manuscript. Figures IVA-D show the differential expression of GR chaperones CHIP, HSP70, HSP90, FKBP51, FKBP52, PTGES, HOP and HIP in HCAE, HCASM, HCM and THP1 cells. The most striking differences were observed for HSP70 expression which was 4-fold higher in HCAEC and 3-fold higher in HCASMC and HCM in comparison to HUVECs and THP1 cells (Figure IV-A and IV-B). Another striking difference was the 2-fold decreased expression

of the constitutive chaperone HSP90 in HCAECs and THP1 cells (Figure IV-A and IV-C). Finally, HCASMCs and HCMs expressed approximately 4-fold higher levels of the GR inhibitor FKBP51, and Dex-mediated upregulation occurred only in Dex-sensitive HUVECs, HCAECs and HCMs (Figure IV-D). Interestingly, the FKBP51 antibody that we used in this study did not recognize the FKBP51 isoforms from THP1, which have been reported in the literature to differ from wild type isoforms (1).

Finally, we performed PCR-RFLP to evaluate the presence of GRα polymorphisms associated with Dex-sensitivity (2-3). Although our study group was small (n=42), we observed similar allele frequencies as previously reported (Table VII). GR nonsynonymous rare alleles were present in less than 5% of our HUVEC pool (ie, 2 heterozygous samples of a total of 42 samples, for both R23K and N363S) and were not associated with Dex-sensitivity (Table VII). Additional direct genomic template DNA sequencing did not reveal any additional nonsynonymous SNPs in our group. Lastly, the common *Bcl*1 polymorphism that is associated with Dex-sensitivity, was not significantly associated with in vitro Dex-sensitivity in our study. Our data suggests that GR polymorphisms are not the key factor in determining GR function in endothelial cells.

References

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Table SI. Primer sequences used for real-time quantitative PCR analysis

Name	Sequence	Reference
GRα	Sense: 5'-CAAAGAGCTAGGAAAAGCCAT-3' Antisense: 5'-CAATACTCATGGTCTTATCCAA-3'	NM_001204264
GRβ	Sense: 5'-TCAGTTCCTAAGGACGGTCT-3' Antisense: ACCACATAACATTTTCATGCAT-3'	NM_001020825
PAI-1	Sense: 5'-GGGCCATGGAACAAGGATGA-3' Antisense: 5'-CTCCTTTCCCAAGCAAGTTG-3'	NM_000602
18S	Sense: 5'-CGGCTACCACATCCAAGGAA-3' Antisense: 5'-GCTGGAATTACCGCGGCT-3'	NR_003286
β-Actin	Sense: 5'-AGAAAATCTGGCACCACACC-3' Antisense: 5'-TAGCACAGCCTGGATAGCAA-3'	NM_001101
ICAM-1	Sense: 5'-AGCTTCTCCTGCTCTGCAAC-3' Antisense: 5'-GTCTGCTGGGAATTTTCTGG-3'	NM_000201
VonWillebrand Factor	Sense: 5'-AGAAACGCTCCTTCTCGATTATTG-3' Antisense: 5'-TGTCAAAAAATTCCCCAAGATACA-3'	NM_000552
Factor VIII	Sense: 5'-ATGCACAGCATCAATGGCTAT-3' Antisense: 5'-GTGAGTGTGTCTTCATAGAC-3'	NM_000132
eNOS	Sense: 5'-GGCATCACCAGGAAGAAG-3' Antisense: 5'-TCACTCGCTTCGCCATCA-3'	NM_000603
VEGF	Sense: 5'-ATGAGGACACCGGCTCTGACCA-3' Antisense: 5'-AGGCTCCTGAATCTTCCAGGCA-3'	NM_001025366

Table II. Clinical Characteristics of the Study Subjects

	Dex-Sensitive (n=30)	Dex-Resistant (n=12)	*P*
Maternal age, y	29.7 ± 7.3	30.4 ± 9.2	0.854
Pre-pregnancy BMI, kg/m^2	25.4 ± 4.5	22.1 ± 3.7	0.046*
Maternal weight gain, lb	32.1 ± 15.5	38.6 ± 10.2	0.223
Systolic blood pressure, mm Hg	115.4 ± 8	104.8 ± 8.7	0.021*
Diastolic blood pressure, mm Hg	72 ± 7.2	67.9 ± 3.7	0.052
Smoking, yes/no	3/30	0/16	0.545
Gestational age (wk)	39.5 ± 1.3	38.7 ± 1.2	0.102
Newborn sex (F/M)	14/16	7/5	0.734
Race, African American/Caucasian (%)	13/30 (43.3)	7/12 (58.3)	0.499
Birth weight (g)	3403 ± 442	3396 ± 472	0.974
Umbilical cord blood cortisol, nmol/L	403 ± 135	412 ± 91	0.833

*p<0.05, Dex-sensitive vs. Dex-resistant.

Table III. Differential response of HUVECs to Dex-mediated endothelial gene regulation

	Dex-Sensitive (n=16)	Dex-Resistant (n=12)
Factor VIII		
Dex 0.1μM	1.25 ± 0.46	0.91 ± 0.59
Dex 1 μM	2.08 ± 0.57*	0.98 ± 0.36†
von Willebrand Factor		
Dex 0.1μM	1.13 ± 0.22	0.78 ± 0.39
Dex 1 μM	2.18 ± 0.92*	0.75 ± 0.38†
ICAM1		
Dex 0.1μM	1.68 ± 0.45*	1.14 ± 0.46
Dex 1 μM	2.98 ± 0.87*	1.41 ± 0.45†
VEGFA		
Dex 0.1μM	0.70 ± 0.33*	1.04 ± 0.26
Dex 1 μM	0.53 ± 0.27*	1.11 ± 0.25†

Values represent the means ± STD of treated versus untreated control levels. *$p<0.05$ Dex-treated vs. untreated †$p<0.05$ Dex-sensitive vs. Dex-resistant.

Table IV. Differential response of HUVECs to Dex-stimulation of GRE-dependent transactivation.

	Dex-Sensitive (n=16)	Dex-Resistant (n=12)
Relative luciferase (fold of Ctl)		
Dex 0.1 μM	5.9 ± 0.7*	2.4 ± 1.4*†
Dex 0.33 μM	6.8 ± 0.9*	2.7 ± 0.7*†
Dex 1 μM	8.0 ± 0.8*	2.9 ± 0.9*†

*p<0.05 Dex-treated vs. untreated †p<0.05 Dex-sensitive vs. Dex-resistant.

Table V. Pro-coagulant activity in Dex-sensitive and Dex-resistant HUVECs.

	Dex-Sensitive (n=10)	Dex-Resistant (n=10)
aPTT (seconds)		
Plasma (no cells)	65.4 ± 2.6	66.1 ± 3.1
+Cells Untreated	55.3 ± 3.3	63.0 ± 4.1†
+Cells Dex 0.33 μM	51.5 ± 3.2	64.3 ± 3.6†
+Cells Dex 1 μM	47.1 ± 2.1*	65.5 ± 4.2†

*p<0.05 Dex-treated vs. untreated †p<0.05, Dex-sensitive vs. Dex-resistant.

Table VI. Basal and Dex-stimulated regulation of GR mRNA levels in HUVECs

	Dex-Sensitive (n=16)	Dex-Resistant (n=12)
Basal mRNA levels (fg GR/pg 18S)		
GRα	12.8 ± 9.1	13.5 ± 9.8
GRβ	0.085 ± 0.092	0.086 ± 0.047
GRα mRNA levels (fold of Ctl)		
Dex 0.1 μM	1.21 ± 0.35	0.92 ± 0.34
Dex 0.33 μM	1.62 ± 0.5*	0.96 ± 0.38
Dex 1 μM	2.06 ± 0.65*	1.07 ± 0.45

*p<0.05 untreated vs. Dex-treated.

Table VII. GR genotypes in Dex-sensitive and Dex-resistant HUVECs

	Dex-Sensitive (n=30)	Dex-Resistant (n=12)	*P*
GR +646 C>G (rs41423247)			
CC	16/30 (53.3%)	5/12 (41.7%)	
CG	10/30 (33.3%)	5/12 (41.7 %)	0.73
GG	4/30 (13.3%)	2/12 (16.7%)	
GR R23K G>A (rs6190)			
GG	28/30 (92.6%)	12/12 (100%)	
GA	2/30 (7.4%)	0/19 (0%)	0.98
AA	0/30 (0%)	0/19 (0%)	
GR N365S A>G (rs1800445)			
AA	28/30 (92.6%)	11/12 (100%)	
AG	2/30 (7.4%)	1/12 (0%)	0.98
GG	0/30 (0%)	0/12 (0%)	

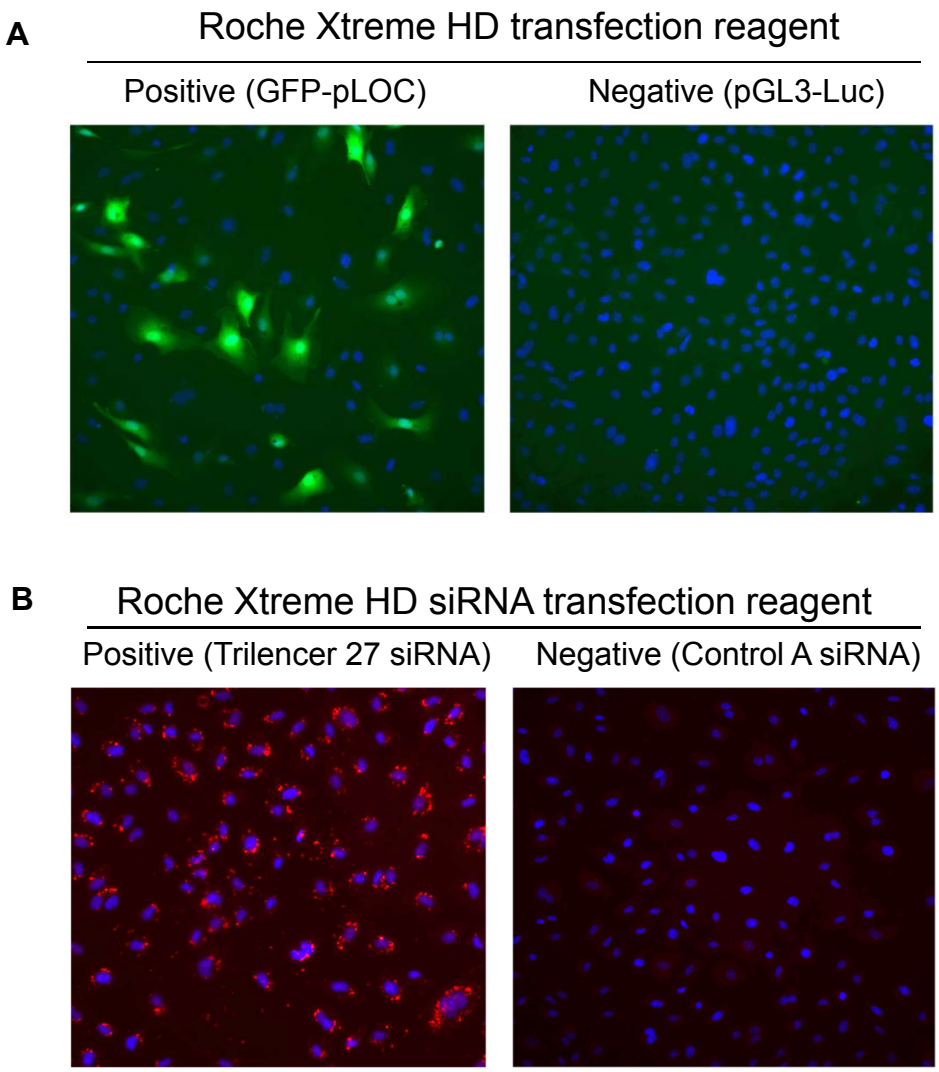

Figure I. DNA vector and siRNA transfection efficiency in HUVECs. A) HUVECs were transfected with Rescue vectors (pLOC) that contain the green fluorescent protein gene, or pGL3-luciferase control vectors using the Xtreme HD reagent to determine the efficiency of GFP gene expression, B) Proliferating HUVECs were transfected with Fluorescent Trilencer 27 (Origene) or Control siRNA (Santa Cruz) using Xtreme HD siRNA reagent. The assays were repeated with 3 different HUVEC samples and a representative figure is shown.

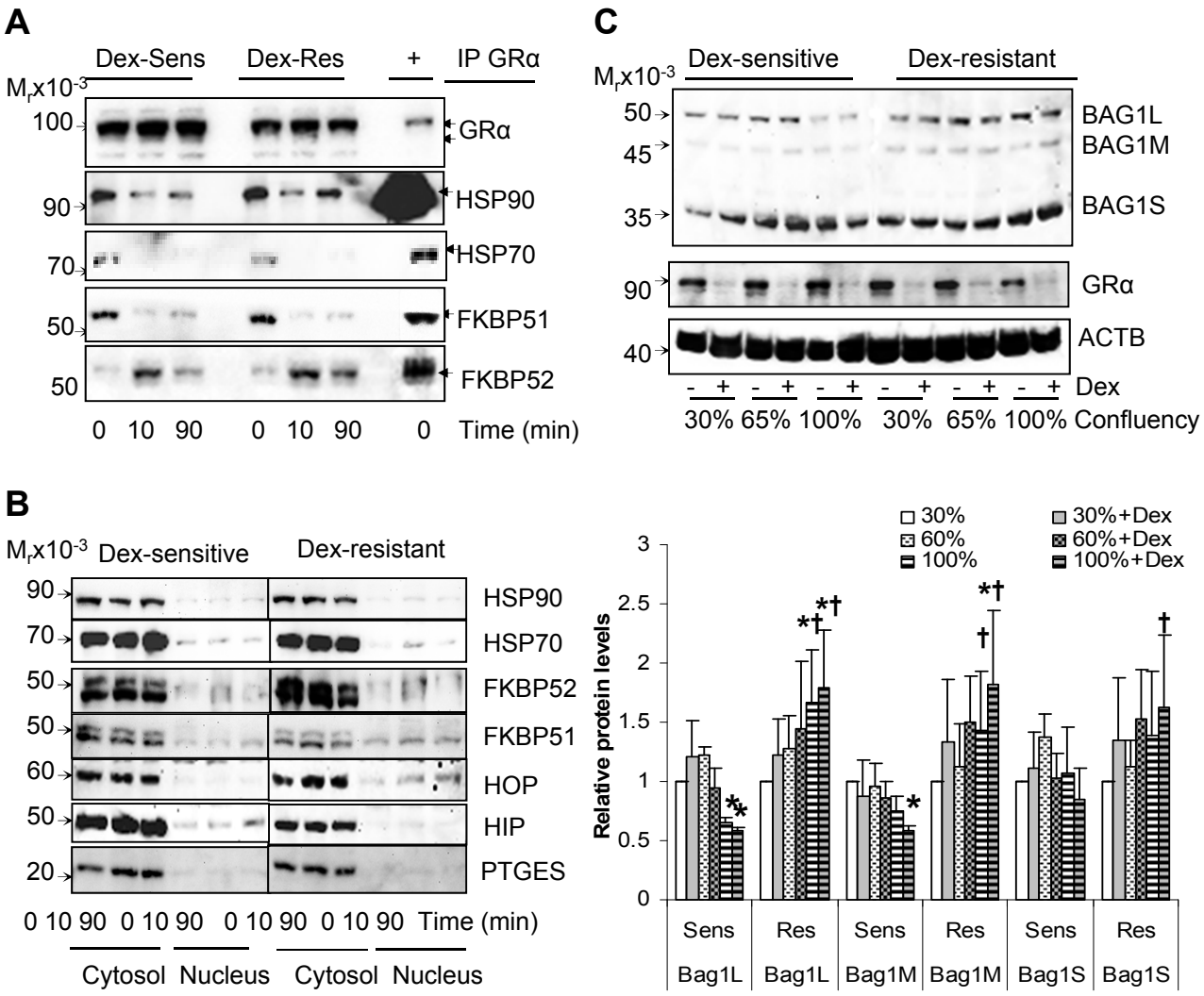

Figure II. Similarities in GR protein regulation between Dex-sensitive and Dex-resistant HUVECs. A) A representative immunoblot shows the similarities in GRα interactions with chaperones HSP90, HSP70, FKBP51 and FKBP52 between Dex-sensitive and Dex-resistant (n=6 each) HUVECs determined via immunoprecipitation. B) Similarities between Dex-sensitive and Dex-resistant HUVECs (n=6 each) in the cytosolic and nuclear expression of GR chaperones HSP90, HSP70, FKBP51, FKBP52, HOP, HIP and PTGES, as shown by a representative immunoblot. C) Expression of GR and BAG1 isoforms in Dex-sensitive and Dex-resistant HUVECs is similar in proliferating conditions. Cells were plated at $1x10^5$, $2x10^5$ and $4x10^5$ to generate cell cultures that were ~30%, 65% and 100% confluent, treated with Dex (1μM) and then analyzed for BAG1 isoform protein expression via immunoblot. Bar graph show the levels of BAG1 isoform, adjusted for ACTB and as a fold of basal levels at 30% confluency. Bars represent the Mean ± STD (n=5/group); * $p<0.05$ vs. 30% confluency, †$p<0.05$, Dex-sensitive vs. Dex-resistant.

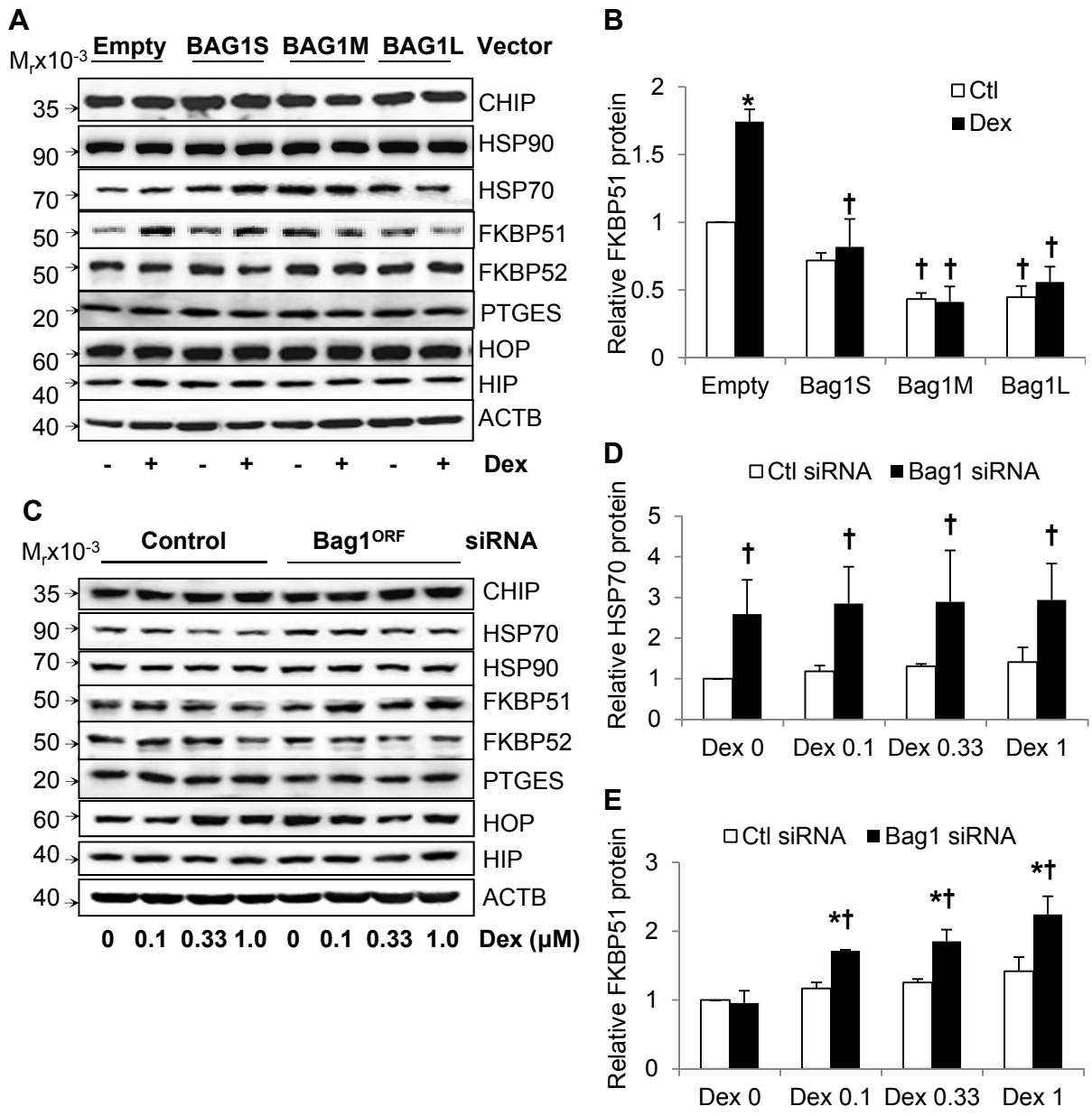

Figure III. Effect of BAG1 overexpression and silencing on GR chaperone expression. A-B) Effect of BAG1 overexpression on GR chaperone expression: Dex-senstive HUVECs were transfected with BAG1 vectors and then exposed to Dex (1μM) for 24 h and protein extracts were analyzed via immunoblotting. A) Representative immunoblots for GR chaperones B) Bar graph for FKBP51 expression. C-E) Effect of BAG1 silencing on GR chaperone expression: Dex-resistant HUVECs were transfected with Control and BAG1^{ORF} siRNA, followed by Dex treatment for 24h and total protein extracts analyzed via immunoblotting. C) Representative immunoblots for GR chaperones, Bar graph showing the effect of BAG1 silencing on HSP70 (D) expression, and FKBP51 (E). Bars represent the Mean ± STD (n=3/group); * $p<0.05$ Basal vs. Dex-treated, †$p<0.05$, Control siRNA/vector versus Bag1 siRNA/vector.

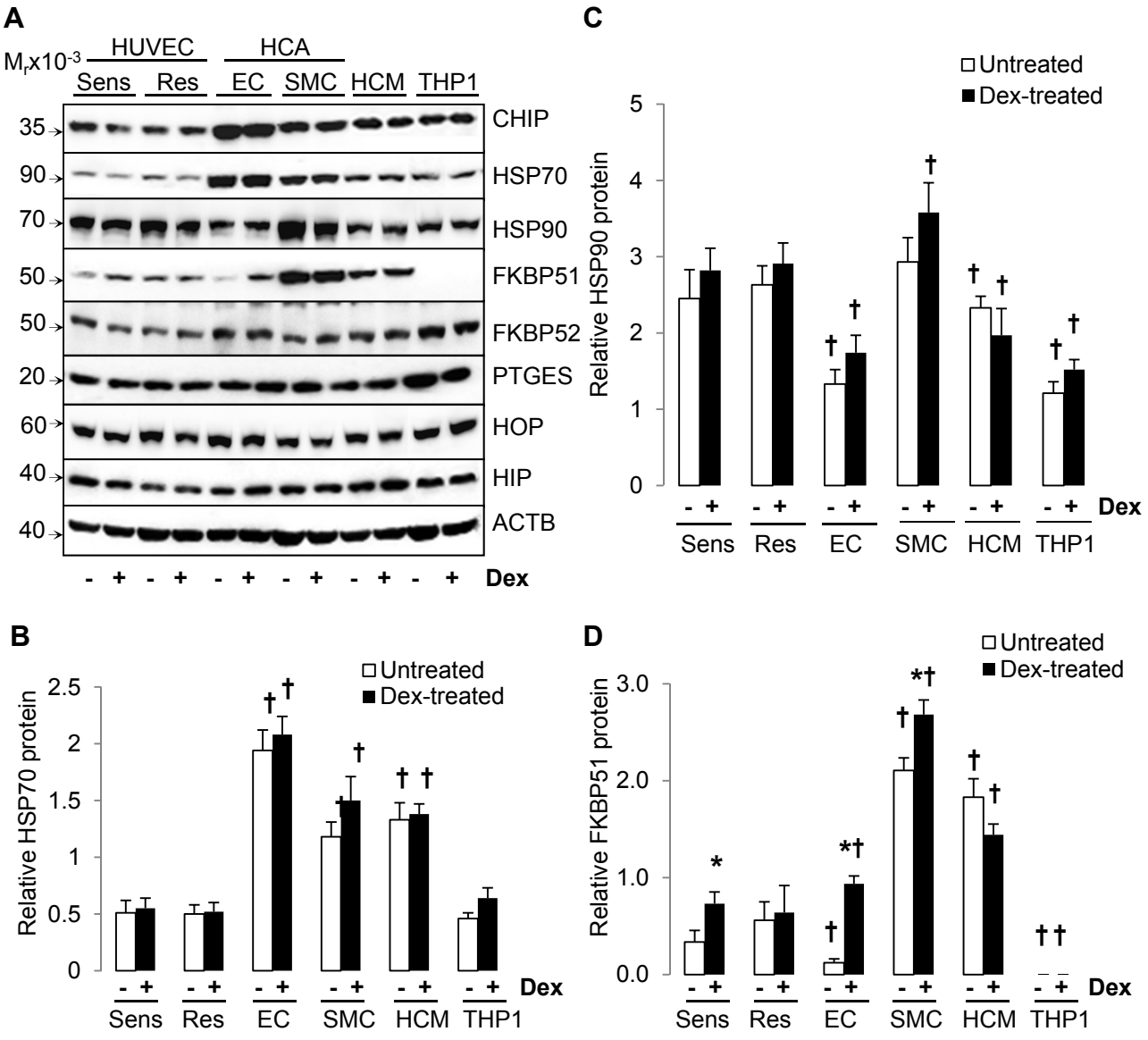

Figure IV. Differential GR chaperone expression in human vascular cells. Human coronary artery endothelial cells (HCAEC), human coronary artery smooth muscle cells (HCASMC), human cardiomyocytes (HCM) and human monocytoid leukemic cells (THP1) were cultured and treated with Dex as described under Methods to examine the expression of the GR chaperone system. A) Representative immunoblots for GR chaperones CHIP, HSP70, HSP90, FKBP51, FKBP52, PTGES, HOP and HIP, B) Bar graph for HSP70 expression, C) Bar graph for HSP90 expression D) Bar graph for FKBP51 expression. Primary cells were tested at different passage number for a total n of 3. Bars represent the Mean ± STD (n=3/group); * $p<0.05$ untreated vs. Dex-treated, †$p<0.05$ vs. untreated Dex-sensitive HUVECs.

Materials and Methods

Subjects and Cell culture-Human umbilical vein endothelial cells (HUVECs) were isolated from 22 Caucasian and 20 African American healthy term pregnancies (50% female newborns). Exclusion criteria were history of present or past hypertension, diabetes, renal disease, cardiovascular disease, coagulopathy or any other vascular or metabolic complication; and fetal distress, congenital disorders or other pregnancy complications. The study was approved by the IRBs of the Loma Linda University and the University of California at San Diego. HUVECs were isolated from a 6-inch umbilical cord segment via collagenase digestion as previously described (1). Cell culture purity was characterized by endothelial cell markers (PECAM1 and von Willebrand Factor) and lack of smooth muscle actin and cell surface fibroblast markers. HUVECs were cultured using EGM-MV bulletkit media (Lonza, MA). All assays were performed between passages 4-6. To study interindividual differences in response to glucocorticoids, we stimulated confluent and quiescent HUVECs with Dex using DMSO as solvent. This synthetic glucocorticoid was chosen for its stability in the presence of cellular 11-beta-dehydrogenase, and its specificity for GR with respect to other nuclear receptors such as the mineralocorticoid receptor (2). To starve HUVECs we used M199 (Sigma Aldrich, St Louis, MO) supplemented with 0.95 mM HEPES, 0.1% BSA, 1% antibiotics and 1% FBS.

Human coronary artery endothelial cells (HCAEC) and human coronary artery smooth muscle cells (HCASMC) were obtained from Lonza (Walkersville, MD) at passage 3. Human primary cardiomyocytes were obtained from ScienCell Res. Lab (Carlsbad, CA). The acute monocytic leukemia cell line THP-1 from Sigma Aldrich (St Louis, MO) is widely used for inflammation research and has been known to respond to Dex (3). Each batch of primary vascular cells was originally derived from a single healthy donor and was cultured according to

the provider's guidelines. HCAECs and HCASMCs were subcultured and tested within 3 different passages, while HCMs were originally cultured and tested in triplicate samples of the same passage because HCMs terminally differentiate in vitro and cannot be subcultured. THP-1 cells were grown in DMEM with 1% antibiotics and 10% FBS.

RNA extraction and real-time PCR: Total RNA was extracted with *Tri*ZOL (Invitrogen, Carlsbad, CA), quantified, and stored at -80°C until analysis. Total RNA (1 μg) was reverse transcribed as described previously (4). Briefly, all PCR reactions were performed in triplicate with SYBR Green and a master mix containing hot-start Taq polymerase (Qiagen, San Diego, CA) and 50 ng of total cDNA equivalent per reaction. PCR was run with denaturation at 95°C for 30 s, annealing at 51-55°C for 30 s, and extension at 72°C for 45 s. The BioRad iCycler equipped with real-time optical fluorescent detection system was used for SYBR Green detection. 18S and β-Actin were used as housekeeping genes. Primers used, together with their accession numbers, are shown in Supplemental table 1. For GRα and GRβ quantification, a standard curve was obtained using hGRα and hGRβ mammalian-expressing vectors (kind gift of Dr. Cidlowski, NIEHS, Research Triangle, NC). Extrapolation of unknowns from the standard curve was performed using Prism 3 (GraphPad Software, San Diego, CA), predicting unknowns from the standard curve C_t values. To obtain relative fold mRNA levels, the delta C_t method was used.

SDS-PAGE and immunoblotting- Western blotting was performed as previously described (4-5). Protein extracts were prepared in cold lysis buffer, or, alternatively, cytosolic and nuclear extracts were prepared using the commercial kit NE-PER (Pierce Biotech., Rockford, IL). Protein samples were heat denatured in Laemmli buffer, separated on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene fluoride membranes.

Membranes were blocked in 5% non fat dried milk in 0.05% Tris-buffered saline (TBST) for 1 h, and then probed in primary antibody overnight at 4 °C. The following antibodies were used: rabbit anti-GRα and anti-GRβ (ABR Affinity Bioreagents, Rockford, IL), monoclonal anti-HSP90, anti-PAI1 and anti-eNOS (BD Biosciences, San Diego, CA); rabbit anti-GR (H-300), rabbit anti-GRα (P-20), mouse anti-BAG1 (F-7) and rabbit anti-CHIP (H-231) (Santa Cruz Biotechnology, Santa Cruz, CA); and monoclonal anti-β-Actin, or ACTB, (Ambion, Austin, TX). To determine the relative abundance of proteins, an internal control (pooled HUVEC extract), and rhGRα (100-500ng, ThermoFisher, Rockford, IL), were used in every membrane. All the antibodies were diluted in blocking buffer containing 5% non-fat dry milk in TBST at a final concentration of 1 μg/ml. After three 10 min washes with TBST, the membranes were incubated with corresponding secondary antibodies that were diluted at 1:2000. Bound antibodies were visualized using the ChemiGlow Chemiluminescent substrate (Alpha Innotech Corp, San Leandro, CA). Digital images were captured using the Alpha Innotech ChemiImager Imaging System with a high-resolution charge-coupled device camera and quantified using the Alpha Innotech ChemiImager 4400 software.

GR protein half-life analysis. To estimate the half-lives of GR protein under basal and Dex stimulation, cells were treated with the translation inhibitor cycloheximide and chase studies were performed as previously described (6). Briefly, confluent and quiescent cells were treated with or without Dex (1 μM) for 1 h. Cycloheximide (10μg/ml) was then added to stop protein translation. Total protein samples were harvested at 0, 4, 8 and 12 and 24 h after cycloheximide treatment for analyzing GRα and ACTB protein levels by immunoblotting as described above.

Analysis of the role of the proteasome in GRα protein degradation. We determined the dose response effect of MG132 (a specific proteosomal inhibitor) on basal and Dex-stimulated GR

protein expression (6). Confluent and quiescent HUVECs (n=5/group) were pre-treated with MG132 (200, 50 and 12.5 nM) for 1 h and then treated with solvent or Dex (1μM) for 18 h and GRα and ACTB protein expression determined via immunoblotting. Data is expressed as relative GRα/ACTB ratios.

Determination of GRα ubiquitination levels and GRα-chaperone interaction by Immunoprecipitation assays. Confluent HUVECs were treated with Dex (1 μM) for various time points and total protein extracts were prepared using a cold non-ionic lysis buffer (10 mM TrisHCl, 100 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton-X100, 0.5% NP-40, 50 mM NaF, 10 mM Na_3VO_4 and Halt protease inhibitors). Aliquots of 200 μg total protein were immunoprecipitated with 2 μg of rabbit polyclonal anti-GR (H-300) antibodies at 4°C overnight. Then 50 μl of protein A-plus-agarose beads (Pierce Biotechnology, Rockford, IL) were added and incubated at 4°C for further 2 h. Immunoprecipitates were washed 3 times with cold lysis buffer, and heat denatured with 1X Laemli buffer containing 0.1M DTT. After SDS-PAGE and electroblotting, membranes were probed using the following antibodies: monoclonal anti-GR, anti-HSP90, anti-PTGE3 and anti-FKBP51 (BD Biosciences, Sparks, MD), rabbit polyclonal anti-HSP70 and anti-HIP (Cell Signaling, Danvers, MA), monoclonal anti-HOP (Stressgen Biotech., San Diego, CA), monoclonal anti-FKBP52 (Abcam, Cambridge, MA), rabbit anti-CHIP and monoclonal anti-BAG1 (Santa Cruz Lab). Densitometries were estimated and relative binding of chaperones to GRα was calculated. To obtain a better representative figure and analyze the interaction of all 3 BAG1 isoforms with GR, we utilized the Direct IP Kit (Pierce ThermoFisher Scientific) to covalently bind 10 μg of purified anti-GR (H-300, Santa Cruz Lab.) antibody to the pureLink resin according to the manufacturer's instructions (7). After preparing the antibody-resin conjugate, GRα protein was immunoprecipitated as described above and

eluted in a low pH buffer, then processed by immunoblotting as described above. This method prevents the elution of the 50kDa antibody protein that interfered with BAG1L and BAG1M analysis, and allowed a better visualization of BAG1 isoforms, FKBP51, and FBKP52. In addition, we included a positive marker, which consists of approximately 10% of the original protein extract.

To determine the levels of GRα ubiquitination with Lysine 48-polyubiquitin chaing that target GR for proteosomal degradation, quiescent and confluent HUVECs were pretreated with 200nM MG132 for 1h, then treated with Dex (1μM) for 2 and 4 h. N-ethylmaleimide (5mM, Sigma Aldrich, MO) was freshly added to the IP lysis buffer to inhibit the cleavage of polyubiquitin chains from their target proteins. Then, Lysine-48 polyubiquitin chains were immunoprecipitated from a 300 μg protein sample, using 2 μg of specific polyclonal rabbit antibody (clone Apu2, Millipore, MA) at 4°C overnight. After a 2h incubation with Protein A-Plus Agarose beads and a quick centrifugation of the beads to pull-down the ubiquitinated proteins, the remaining protein sample (supernatant) was transferred to another vial and incubated with 2 μg of rabbit polyclonal anti-GR (H-300) and the non-ubiquitinated GR protein was then immunoprecipitated as described above. Eluates from the Lys-48 polyubiquitin IP and the GR-IP were processed by immunoblotting and probed for GR protein levels using the BD monoclonal anti-GR antibody. The original protein lysates were also analyzed for the levels of Lysine 48-polyubiquitinated chains and monoubiquitin protein levels by immunoblot. Polyubiquitinated GR bands were observed at 90-120 kDa, while non-ubiquitinated GR molecules were observed at 80-95 kDa. Results are expressed as the percentage of ubiquitinated GR over total GR. A positive marker (10% original protein lysate) was included. A total of 5 different sets of Dex-sensitive and Dex-resistant HUVECs were analyzed.

Cell transfection and luciferase assays –Transfection of luciferase vectors was performed with the aid of HD Xtreme transfection reagent (Roche, CO). Luciferase assays were performed as previously described (8). Briefly, confluent cells were transfected with the minimal GRE-luciferase construct (Clontech, Palo Alto, CA) using a 1:1 complex of DNA:Xtreme reagent and according to the manufacturer's protocol. TK-Renilla luciferase vector (Promega Corp, Rockford, IL) was used as the internal control. The transfection was carried out at 37°C for 6 h. The cells were allowed to recover in complete culture medium for 18-20 h, and then treated with starvation media with or without Dex for another 24 h. Firefly and Renilla luciferase activities were measured using a dual-reporter assay kit (Promega) according to the manufacturer's protocol. Relative luciferase values were calculated as a ratio of firefly/ renilla luciferase activities. Each treatment was tested in quadruplicates and repeated twice using different cell passages.

BAG1 silencing, overexpression and rescue siRNA assays. To overexpress BAG1, we obtained mammalian expression vectors (kind gift of Dr. John C. Reed, Burnham Institute, La Jolla, CA) that contain the human BAG1 long (BAG1L, 50kDa), medium (BAG1M, 46kDa) and short (BAG1S, 36kDa) forms. HUVECs do not express the shortest BAG1 form (p29), therefore, this isoform was not studied. Subconfluent HUVECs, and HCASMCs (60-80%) were transfected with Xtreme HD transfection reagent (Roche), as described above, with the exception that SMC required a ratio of 1:2 of DNA:reagent instead of 1:1. The efficiency of our transfection protocol was approximately 40% as determined by the expression of green fluorescent protein (Supplemental Figure 1A). Overexpression of BAG1 isoforms was confirmed by immunoblotting. HUVECs and HCASMCs overexpressed all isoforms in the order of M>S>L.

To silence BAG1 expression, we utilized a commercially available pool of 4 BAG1 siRNAs that target the open reading frame (ORF) of BAG1 (Santa Cruz Lab). Subconfluent (70%) HUVECs were transfected with Xtreme HD siRNA transfection reagent (Roche Diagnostics, Boulder, CO) using a RNA:reagent ratio of 1μM:10 μL and according to the manufacturer's protocol. To determine the efficiency of our transfection protocol, we transfected a Trilencer-27-fluorescent control siRNA (Origene) and determined that our protocol yields approximately 85% of positively transfected cells (Supplemental Figure 1B). We identified that 6 μl of BAG1 siRNA (0.5 μM) was sufficient to inhibit BAG1L and BAG1S protein expression by more than 50% of basal levels.

To confirm our overexpression and silencing results, we performed rescue siRNA assays as previously described (9). We obtained control and BAG1 expressing vectors (pLOC vectors, Open Biosystems, ThermoFisher) that express the Blastocidin S resistance gene in order to generate stably transfected cells. Transfected cells were selected in culture media containing 5 μg/mL Blasticidin S over 2 weeks of culture. Non-transfected cells died within 5 days of culturing in Blasticidin S media. Selected cells expressed rescue BAG1 isoforms derived from an artificial ORF mRNA that lacked non-coding segments. In addition, we designed 6 novel $BAG1^{3'UTR}$ siRNAs (Dharmacon RNAi Technologies, Thermo Fisher). After optimization, we chose a combination of 3 $BAG1^{3'UTR}$ siRNAs: BAG1 1742 bp (GGAUGGAGCCUGUGGUUGA), BAG1 2653 bp (CGCUAUAACUCUACCUAAA), and BAG1 3016 bp (GCGCAAGGUUGUAGAGUAA). These $BAG1^{3'UTR}$ siRNAs were used to transfect stably transfected HUVECs at a final concentration of 0.6 μM total (0.2μM each siRNA), which decreased BAG1L expression by 70% and BAG1S expression by 30% of basal

levels. A total of 4 DEX-resistant and 3 DEX-sensitive HUVECs were tested in rescue assays to confirm our results on the role of BAG1 in GR protein turnover and function.

Procoagulant Activity Assay. The procoagulant activity of HUVECs was tested by a one-step recalcification time test, also known as the activated partial thromboplastin time (aPTT) as previously described (10). Confluent and quiescent HUVECs were treated for 24h with a solvent only, or Dex (0.33 and 1 uM). Cells were then washed, trypsinized and resuspended in PBS at a concentration of one million cells per mL PBS. A 1:1 solution was prepared with cells and fresh plasma (collected from one single donor and anticoagulated with 3.8% sodium citrate, 1:9, v/v) and incubated for 180 s at 37°C. After the addition of preheated 25 mmol/L $CaCl_2$, the time to fibrin strand generation was recorded by a BCS XP hemostatic analyzer (Siemens, Munich, Germany).

Genotyping. Restriction fragment-length polymorphism (RFLP) was performed to determine NR3C1 genotypes as described previously (11). Initially, 20 μL of relevant PCR product was digested by 10 units of *Bcl*I restriction endonuclease to determine rs 41423247, by 10 units of *Tsp*5091 restriction endonuclease to determine rs1800445; and by 10 units of *Mn*II to determine rs6190. All digestions were performed at 37ºC overnight and separated in 2% agarose gels with gel-star staining to visualize the bands. To confirm the accuracy of the genotyping performed by PCR-RFLP, 3 randomly selected samples from each genotype was analyzed by direct DNA sequencing using the ABI 310 Genetic Analyzer (Applied Biosystems, Carlsbad, California). No discrepancies were found. In addition to GR, BAG1 coding sequences (accession number NM_004323) were analyzed by direct DNA sequencing as described above.

Statistical analysis- Data are presented as means ± STD. Differences between two groups (i.e., Dex-sensitive versus Dex-resistant, and Basal versus Dex-treated) was analyzed by Student's *t*-

test. Group distribution differences were analyzed by Fisher's exact method and genotype distribution differences by Pearson's method. A p value of less than 0.05 was regarded as significant. All statistical analysis was performed using SPSS, version 16.

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