ORIGINAL RESEARCH

Uremic Advanced Glycation End Products and Protein-Bound Solutes Induce Endothelial Dysfunction Through Suppression of Krüppel-Like Factor 2

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Background—Cardiovascular disease is the leading cause of morbidity and mortality in patients with end-stage renal disease. The accumulation of uremic solutes in this patient population is associated with endothelial dysfunction and accelerated cardiovascular disease. In this study, we examined the impact of the uremic milieu on the endothelial transcription factor, Krüppel-like factor 2 (KLF2), a key regulator of endothelial function and activation.

Methods and Results—Using serum from uremic pigs with chronic renal insufficiency, our results show that KLF2 expression is suppressed by the uremic milieu and individual uremic solutes in vitro. Specifically, KLF2 expression is significantly decreased in human umbilical vein endothelial cells after treatment with uremic porcine serum or carboxymethyllysine-modified albumin, an advanced glycation end product (AGE) known to induce endothelial dysfunction. AGE-mediated suppression of KLF2 is dependent on activation of the receptor for AGE, as measured by small interfering RNA knockdown of the receptor for AGE. Furthermore, KLF2 suppression promotes endothelial dysfunction, because adenoviral overexpression of KLF2 inhibits reactive oxygen species production and leukocyte adhesion in human umbilical vein endothelial cells. In addition, the application of hemodynamic shear stress, prolonged serum dialysis, or treatment with the receptor for AGE antagonist azeliragon (TTP488) is sufficient to prevent KLF2 suppression in vitro. To decipher the mechanism by which uremic AGEs suppress KLF2 expression, we assessed the role of the receptor for AGE in activation of nuclear factor-κB signaling, a hallmark of endothelial cell activation. Using a constitutively active form of IκBα, we show that translocation of p65 to the nucleus is necessary for KLF2 suppression after treatment with uremic AGEs.

Conclusions—These data identify KLF2 suppression as a consequence of the uremic milieu, which may exacerbate endothelial dysfunction and resultant cardiovascular disease. **(*J Am Heart Assoc*. 2018;7:e007566. DOI: 10.1161/JAHA.117.007566.)**

Key Words: advanced glycosylation end products • chronic kidney disease • endothelial dysfunction • Krüppel-like factor 2 • uremia

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in patients with chronic and end-stage renal disease (ESRD). Even after adjusting for traditional risk factors, uremic patients requiring dialysis have approximately a 15-fold higher risk of cardiovascular mortality compared with the general population.[1,2] It has been suggested that the accumulation of uremic solutes in patients with ESRD augments CVD.[3] Although these toxic solutes range from small to large structures, recent focus has elucidated a role for protein-bound solutes, which are poorly eliminated by conventional dialysis.[4,5] These toxins have been shown to induce vascular inflammation, endothelial dysfunction, and oxidative stress involved in the pathogenesis of CVD.[6–8]

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Clinical Perspective

What Is New?

- Uremic advanced glycation end products and other toxins that accumulate in end-stage renal disease lead to suppression of the endothelial transcription factor Krüppel-like factor 2.
- Suppression of Krüppel-like factor 2 by the uremic milieu exacerbates endothelial dysfunction in atherosclerotic-prone regions.

What Are the Clinical Implications?

- Therapies that improve Krüppel-like factor 2 expression could be beneficial in preventing cardiovascular disease in chronic kidney disease.

Endothelial dysfunction is thought to play a major role in the increased incidence of CVD in ESRD.[9,10] Patients with chronic kidney disease (CKD) show early signs of endothelial dysfunction, which manifests as decreased NO availability, increased oxidative stress, inflammatory activation, and eventual apoptosis or necrosis.[11–14] In the setting of ESRD, the endothelium is under constant exposure to the uremic milieu, making uremic solutes good candidates as mediators of endothelial dysfunction. Previous studies have shown that the protein-bound solutes indoxyl sulfate (IS) and p-cresol sulfate (PCS) are capable of inhibiting cellular proliferation and inducing reactive oxygen species (ROS) production.[15–17] In addition, protein-bound advanced glycation end products (AGEs) up-regulate the expression of endothelial adhesion molecules and inhibit NO production.[18–20] These results suggest that protein-bound uremic solutes contribute to the high prevalence of endothelial dysfunction in patients with ESRD. However, although endothelial dysfunction in uremia has been well characterized, the precise molecular mechanisms by which these solutes inhibit endothelial function remain unclear.

The endothelial transcription factor, Krüppel-like factor 2 (KLF2), is a key regulator of vascular development and homeostasis.[21,22] Within the vessel wall, KLF2 is selectively expressed in the endothelium and is responsible for altering gene transcription in response to hemodynamic shear stress.[22–24] KLF2 is induced by laminar shear stress and statins, resulting in the expression of vasoprotective mediators, such as endothelial NO synthase, thrombomodulin, and the antioxidant transcription factor nuclear factor erythroid-2.[25] In contrast, KLF2 is inhibited by turbulent flow or inflammatory cytokines, leading to opposing effects. At the tissue level, KLF2 expression negatively correlates with the formation of atherosclerosis and neointimal hyperplasia in both arteries and veins.

Given that expression of KLF2 plays a key role in regulating endothelial cell function, we investigated whether uremic serum and protein-bound uremic solutes induce endothelial dysfunction through suppression of KLF2 in vitro. We recognized that the in vivo impact of such findings could play a role in the pathogenesis of both the accelerated vascular disease and aggressive dialysis vascular access stenosis experienced by patients with advanced CKD and ESRD.

Methods

Chemicals and Reagents

The data, methods, and materials that support the findings of this study are available on reasonable request. Tumor necrosis factor α was purchased (RND Technologies) and used at a final concentration of 10 ng/mL for all treatments. Angiotensin II and IS were obtained from Sigma. PCS was purchased from AlsaChim (catalog no. C3302). KLF2 and green fluorescent protein (GFP) adenoviral constructs were a kind gift from Dr Mukesh Jain (Case Western Reserve University, Cleveland, OH). IκBα dominant-negative mutant (IκBα-S32A/S36A) was purchased from Vector Biolabs.

Uremic Porcine Serum

Chronic renal insufficiency was induced in Yorkshire pigs through a total nephrectomy of the right kidney, followed by selective ligation of the vascular supply of the contralateral kidney, as previously described by our group.[26] Blood samples were collected sequentially over a 42-day period and analyzed for markers of renal insufficiency. Animals with renal insufficiency had an initial increase in creatinine to >8 mg/dL, followed by a stabilization between 3 and 4 mg/dL by 2 weeks. After 6 weeks, blood was collected from each animal, separated into uremic serum aliquots, and frozen at −80°C until use. Normal pig serum samples without renal insufficiency were used as controls. For experiments, serum aliquots from multiple animals were pooled and diluted to a final concentration in prepared M200 media. All procedures and experiments were approved by the institutional animal care and use committee at the University of Cincinnati and University of Arizona.

AGE Preparation

Carboxymethyllysine-modified BSA (CML-BSA) was prepared as previously described.[27] Briefly, 5.18 mg of glyoxylic acid (70 nmol) was added per 1 mL of BSA dissolved in PBS at a concentration of 20 mg/mL (glyoxylic acid/albumin lysine

molar ratio of 4:1). The pH was adjusted to 7.4 with NaOH, and 11.06 mg of sodium cyanoborohydride (176 nmol; Sigma) was added per 1 mL of solution. The mixture was incubated for 24 hours at 37°C in a shaking incubator. Control proteins were prepared under the same conditions without glyoxylic acid. Preparations of CML-BSA or unmodified BSA were then extensively dialyzed versus PBS to remove unreacted reagents. The percentage of lysine residues modified to carboxymethyllysine in each preparation was determined by 2,4,6-trinitrobenzene sulfonic acid (Pierce) compared with an L-lysine standard.[28-29] Carboxymethyllysine concentration was quantified by ELISA (CellBioLabs) and assessed for endotoxin contamination before experiments by the Limulus Amebocyte lysate assay (Pierce). The level of endotoxin in all preparations was <30 IU/mL.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were acquired from Fisher Scientific and cultured in Medium 200 (Cascade Biologics) with low-serum growth supplement, 100 μg/mL streptomycin, and 100 IU/mL penicillin at 37°C with 5% CO_2, as previously described.[30] Cells were used between passages 3 and 5 for all experiments. THP-1 monocytes (ATCC) were maintained under standard cell culture conditions in RPMI 1640 medium supplemented with 10% fetal bovine serum, 25 mmol/L HEPES, 1 mmol/L sodium pyruvate, 1 g/L D-glucose, and 1% penicillin/streptomycin.

For experiments, cells were serum deprived for 4 hours and incubated with the different treatments at variable concentrations and times (see figure legends). IS and PCS were dissolved in sterile saline to produce concentrated 30× stock solutions. Experiments with these 2 toxins were conducted in media supplemented with BSA at the average uremic concentration of 35 g/L to account for the high protein binding of these metabolites.[31] Treatments with CML-BSA and control BSA AGE residues, described previously, were performed in complete M200 media.

Assembly of Orbital Shear Rings and Culture Under Shear Stress

Orbital shear rings were constructed as previously described.[32] Shear rings were coated with 1% gelatin, and HUVECs were cultured until confluent. Before experiments, cells were preconditioned for 72 hours under an arterial shear stress of 11 dyne/cm^2 on an orbital shaker (200 rpm) or static culture. The maximum shear stress within the shear ring was estimated according to the following equation:

$$\tau_{max} = r\sqrt{\mu\rho(2\pi f)^3}$$

where τ_{max} is the maximum shear stress, r is the radius of rotation for the orbital shaker (in cm), μ is the viscosity of the medium (in poise), ρ is the density of the medium (in g/mL), and f is the frequency of rotation (in rotation/s).[33] Cell culture and treatments were performed as previously described.

Cell Viability and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assays

HUVEC proliferation was measured using the CellTiter 96 One Cell Proliferation Assay Kit (Promega), according to the manufacturer's instructions. Cells were plated at a density of 3×10^3 cells per well in a 96-well plate. The following day, cells were serum starved for 4 hours and then treated with nonuremic or uremic porcine serum at multiple concentrations for 48 hours. Next, 20 μL of CellTiter 96 AQueous One Solution Reagent was added to each well and incubated at 37°C for 2 hours. The amount of soluble formazan produced by cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was then measured using a plate reader (Biotek) at an absorbance of 490 nm. Percentage viability was calculated by dividing the mean absorbance of treated cell/untreated cells.

Cellular apoptosis was measured using the Click-iT terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) Alexa Fluor 488 Imaging Assay (catalog no. C10245; Invitrogen), following the manufacturer's instructions. Briefly, HUVECs were treated in 96-well plates with nonuremic or uremic porcine serum for 24 hours at concentrations ranging from 0% to 100%. Cells were then washed in PBS and fixed in 4% paraformaldehyde for 15 minutes. Cells were permeabilized with 0.25% Triton X-100, and DNAse I was used as a positive control in untreated cells. After incubation with terminal deoxynucleotidyl transferase (TdT) reaction buffer, cells were counterstained with Hoechst 33342 and imaged on a Cytation5 (Biotek) plate reader. The numbers of positive and total cells were counted in 16 high-power fields for each well and averaged for each treatment group. Results were reported as the percentage of positive cells.

Small Interfering RNA and Adenovirus Transfections

Human receptor for AGE (RAGE) small interfering RNA and a scrambled control small interfering RNA were purchased from Integrated DNA Technologies and transfected using Lipofectamine LTX with Plus reagent and Opti-MEM medium, following the manufacturer's protocol. For KLF2 overexpression studies, cells were transduced with recombinant

adenovirus expressing KLF2 and GFP, GFP, or IκBα dominant-negative mutant at a multiplicity of infection of 10. Briefly, HUVECs were seeded in 24-well plates at a density of 1.5×10^4 cells/cm^2 24 hours before transfection. Transfection efficacy was assessed after 48 hours with Hoechst live-cell stain using a Cytation 5 plate imager (Biotek). Transfection efficacy was >95% in all experiments.

Western Blot and In-Cell Western Analyses

HUVECs were lysed in M-PER buffer or NE-PER buffer (Fisher Scientific), following the manufacturer's instructions, with the addition of protease inhibitors. For immunoblot studies, protein was separated on 4% to 20% Tris-glycine gel and transferred onto a nitrocellulose membrane. Membranes were incubated with Odyssey blocking buffer (Li-Cor) before incubation with anti-KLF2 (Santa Cruz), anti–β-tubulin (Santa Cruz), anti-p65 (Cell Signaling Technologies), or anti-actin (Sigma) primary antibodies. Secondary donkey anti-goat IgG 800CW and/or donkey anti-rabbit 680RD were applied for 90 minutes at room temperature (1:20 000; Li-Cor). For in-cell protein analysis, treated cells were fixed on plates in 4% paraformaldehyde for 20 minutes at room temperature. Antibody treatments, washings, and quantification were performed as previously described.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from cultured HUVECs by the use of TRIzol and the RNeasy mini kit (Qiagen), according to the manufacturer's instructions, with the addition of DNase treatment to eliminate any genomic DNA. First-strand cDNA was synthesized using the High-Capacity RNA-to-cDNA kit (Life Technologies). Quantitative real-time PCR was performed using TaqMan primers and master mix (Life Technologies) and the StepOnePlus RT-PCR machine (Life Technologies). Relative expression was normalized to 18s rRNA as an internal control. Results were evaluated using the ΔΔCt method and expressed as a fold change compared to controls.

Monocyte Adhesion Assays

Adhesion of THP-1 cells to treated HUVECs was measured as previously described.[34] Briefly, THP-1 cells were washed with RPMI medium and labeled with Calcein-Red-Orange (Molecular Probes) at a final concentration of 10 μmol/L. After 3 washes, THP-1 cells were diluted in culture media, and 50 000 cells were added to each well of HUVECs for 30 minutes. Total fluorescence intensity (Ft), automated THP-1 cell counts, and fluorescence of blank media (Fb) were measured with a Cytation 5 plate reader. Unbound THP-1 cells were removed by 3 washings, and the remaining fluorescence intensity (Fx) and THP-1 cell counts were measured again. Percentage adhesion was calculated by the following formula and confirmed with cell counts:

$$\%\text{Adhesion} = (\text{Fx} - \text{Fb})/(\text{Ft} - \text{Fx}) \times 100$$

Detection of ROS

ROS production was measured in HUVECs transfected with KLF2 overexpression adenovirus (recombinant adenovirus expressing KLF2 and GFP) or empty vector (recombinant adenovirus expressing GFP) by incubating cells with the CellROX Orange Reagent fluorescent probe (Molecular Probes) and Hoechst live-cell stain after treatment for 24 hours with uremic serum or toxins. Probes were added to the complete media and incubated at 37°C for 30 minutes. ROS fluorescence was visualized and quantified in living cells using a Cytation 5 plate reader (excitation/emission wavelengths, 545/565 nm). Fluorescent intensity of each cell was measured in 16 high-power fields for each well and averaged across the total number of cells in each treatment group.

Statistical Analysis

All bar and line graphs, as well as statistical analysis, were performed with the Prism 6.0 software (GraphPad Software Inc, San Diego, CA). Unless otherwise indicated, data are reported as mean±SEM. For 2-group comparisons of parametric data, a Student *t* test was performed, whereas nonparametric data were analyzed with a Mann-Whitney rank-sum test. Statistical significance between multiple groups was assessed by 1-way ANOVA on ranks with a Dunn post hoc test (nonparametric), 1-way ANOVA with a Holm-Sidak post hoc test (parametric), or 2-way ANOVA with a Holm-Sidak post hoc test, where appropriate. $P<0.05$ was considered statistically significant.

Results

Uremic Porcine Serum Decreases Endothelial Cell Viability in Vitro

Although the use of pooled serum from uremic patients has been beneficial in describing the impact of uremia on immediate endothelial cell reactivity and survival, the presence of medications, risk factors, or other disease states in the serum of patients with advanced CKD and ESRD could also alter endothelial function and confound the specific impact of uremia. To account for these variables, we used a porcine model of chronic renal insufficiency to replicate the uremic milieu. Uremic serum was obtained from a recently described uremic pig model with documented elevations of creatinine. Consistent with patients with advanced CKD and

ESRD, serum concentrations of blood urea nitrogen and creatinine were significantly elevated between 34 and 43 mg/dL and between 4.5 and 5.8 mg/dL, respectively, in uremic pigs at 6 weeks postoperatively (data not shown). The use of uremic serum allowed us to quantify the impact of all uremic mediators (both known and unknown) in our model systems.

To test the impact of the uremic milieu on endothelial proliferation and survival, we incubated HUVECs with increasing concentrations of pooled uremic or nonuremic porcine serum. Increasing concentrations of uremic porcine serum resulted in reduced endothelial cell viability compared with nonuremic serum (Figure 1A). At a concentration of 20% uremic serum, the impact on HUVEC viability was pronounced compared with nonuremic serum (31±1.4% versus 43±3.7%; P=0.002). This was maintained at higher concentrations. We then measured the induction of apoptosis in serum-treated cells via a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. HUVECs treated with increasing concentrations of uremic serum had a higher percentage of apoptotic cells compared with nonuremic serum (Figure 1B). Incubation with 20% uremic serum resulted in significant apoptosis compared with nonuremic serum (43±0.7% versus 37±0.8%; P<0.0001) and almost complete cell death at higher concentrations.

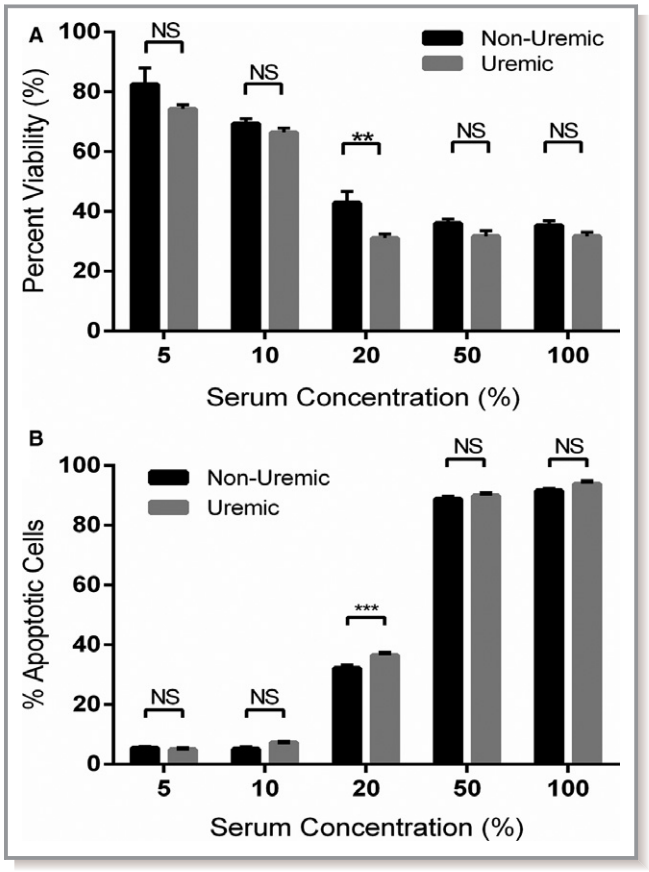

Figure 1. Uremic porcine serum alters endothelial cell viability and promotes apoptosis. A, Human umbilical vein endothelial cells (HUVECs) were incubated in nonuremic porcine serum or uremic porcine serum at multiple concentrations for 24 hours, and cell viability was assessed using the Celltiter One Proliferation Assay. Cell viability is expressed as a percentage compared with untreated cells. B, HUVECs were incubated in nonuremic porcine serum or uremic porcine serum at multiple concentrations, and 24 hours later, the percentage of apoptotic cells was determined via fluorescent terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. Histobars represent mean±SEM, n=8 per group (repeated 3 separate times). NS indicates nonsignificant. **P<0.01, ***P<0.001 (2-way ANOVA with Holm-Sidak post hoc test).

Uremic Porcine Serum Suppresses Endothelial KLF2 Expression

To investigate if uremic solutes alter endothelial KLF2 expression, we first incubated HUVECs with nonuremic or uremic porcine serum over a 24-hour interval. Because endothelial cell viability and apoptosis were pronounced at serum concentrations >20%, we used 10% uremic serum for these studies. Incubation of HUVECs with uremic serum led to decreased KLF2 transcription after 12 and 24 hours compared with baseline (0.36±0.11 and 0.22±0.07; P<0.01; Figure 2A). Similarly, uremic serum significantly suppressed KLF2 protein expression after 24 hours compared with nonuremic serum (0.43±0.14 versus 1.18±0.35; P=0.0005; Figure 2B). However, higher concentrations (20%) of either serum (uremic or nonuremic) resulted in near complete loss of KLF2, likely because of the high rate of cell death.

Uremic Suppression of KLF2 Is Reversed by Dialysis and Shear Stress

Endothelial expression of KLF2 is highly upregulated under laminar flow conditions and inhibited by proinflammatory stimuli.[22] To test the hypothesis that KLF2 suppression is attributable to the presence of uremic solutes in states of chronic renal insufficiency, we extensively dialyzed uremic porcine serum for 24 hours against PBS using a membrane with a molecular weight cutoff of 12 kD. HUVECs were then incubated with 10% uremic porcine serum up to 48 hours and switched to dialyzed porcine serum for an additional 24 hours. KLF2 expression was significantly reduced through 48 hours of treatment with uremic serum. After replacement of the culture media with dialyzed serum, KLF2 expression returned to baseline levels (0.48±0.06 versus 1.01±0.04; P<0.0001; Figure 2D). These results indicate that differential concentrations of uremic molecules present in the serum are likely responsible for the transient changes in KLF2 expression, with prolonged dialysis significantly reducing uremic solutes (free and bound) in the serum.

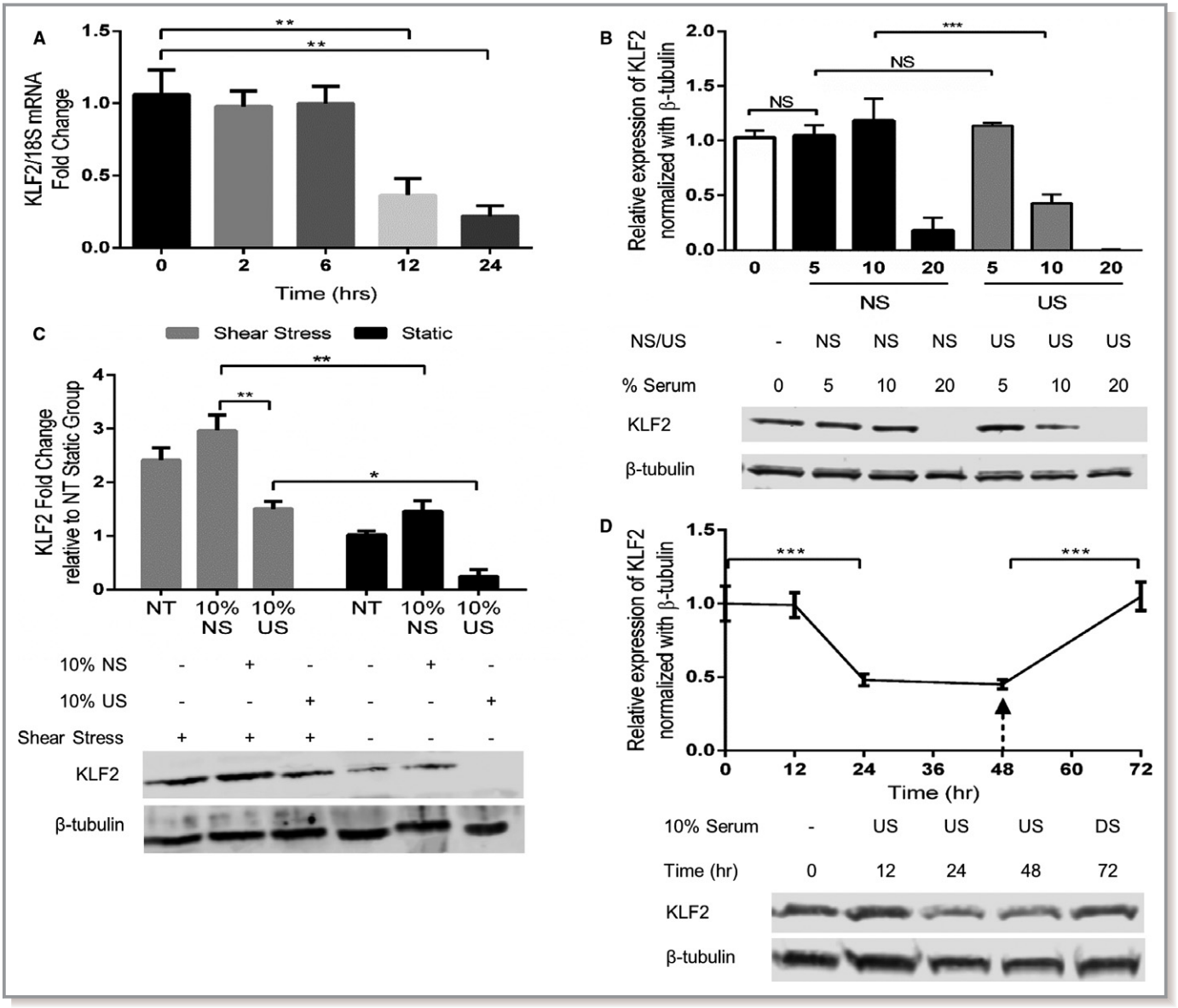

Figure 2. Uremic porcine serum suppresses endothelial Krüppel-like factor 2 (KLF2) expression, which is attenuated by hemodynamic shear stress and dialysis. A, Changes in KLF2 mRNA expression were assessed over a 24-hour time course by quantitative real-time PCR in human umbilical vein endothelial cells (HUVECs) incubated with 10% uremic porcine serum. One-way ANOVA with the Dunn post hoc test was used to calculate statistical significance between treated cells at each time point (n=4 per group; repeated 3 separate times). B, Western blot analysis and quantification of KLF2 from HUVECs incubated with increasing concentrations of normal porcine serum (NS) or uremic porcine serum (US) for 24 hours; basal medium was used as a control (n=4 per group; repeated 3 separate times). C, Western blot analysis and quantification of KLF2 from HUVECs cultured under periodic unidirectional flow using orbital shear rings. Cells were grown to confluence in shear rings and preconditioned for 72 hours under shear stress of 11 dyne/cm^2 on an orbital shaker (200 rpm) or static culture. Cells were then treated as in B and compared to untreated cells (NT) (n=4 per group; repeated 3 separate times). D, Western blot analysis and quantification of KLF2 from HUVECs incubated with 10% uremic porcine and then switched to dialyzed porcine serum (DS) after 48 hours (n=4 per group; repeated 3 separate times). Arrow marks time of switch from uremic to dialyzed serum. Quantification of protein expression above each blot is relative to baseline or untreated cells and normalized to β-tubulin expression. Two-way ANOVA with the Dunn post hoc test was used to calculate statistical significance between serum type and concentrations. Data presented as mean±SEM. NS indicates nonsignificant. *P<0.05, **P<0.01, ***P<0.001.

To assess whether the response of KLF2 to uremic stimuli could be compensated for by changes in vascular shear stress, we incubated HUVECs under periodic unidirectional flow using orbital shear rings.[32] Cells were preconditioned for 72 hours under an arterial shear stress of 11 dyne/cm^2 on an orbital shaker before treatment with 10% uremic or nonuremic porcine serum and assessed for KLF2 expression (Figure 2C). The application of periodic shear stress significantly increased KLF2 expression in untreated cells compared with static culture (2.4±0.2 versus 1.0±0.2; P=0.003). After

incubation with uremic serum, shear stress also prevented the suppression of endothelial KLF2 compared with static culture (1.5±0.1 versus 0.3±0.1; P=0.007). These findings suggest that uremia may exacerbate CVD or dialysis vascular access dysfunction in regions predisposed to low or oscillatory shear stress by enhancing the suppression of endothelial KLF2.

AGEs Are a Potent Inhibitor of KLF2 Expression in Uremia

To investigate whether specific protein-bound uremic solutes are responsible for altering endothelial KLF2 expression, we incubated HUVECs with individual protein-bound uremic toxins likely to be present in uremic serum, at an average concentration found in ESRD, including the following: CML-BSA (5.4 mg/L), IS (23.1 mg/L), and PCS (20.9 mg/L).[35] CML-BSA and PCS both significantly reduced KLF2 expression compared with vehicle (BSA) treated cells after 24 hours (Figure 3A). More important, carboxymethyllysine concentrations in our uremic porcine serum (5.7–8.2 μg/mL) were similar to human ESRD.

Given the response of endothelial cells to CML-BSA and the strong evidence linking AGE/RAGE signaling in endothelial dysfunction, we further examined the action of AGEs on endothelial KLF2 and on KLF2 functionality. Incubation of HUVECs with increasing concentrations of CML-BSA resulted

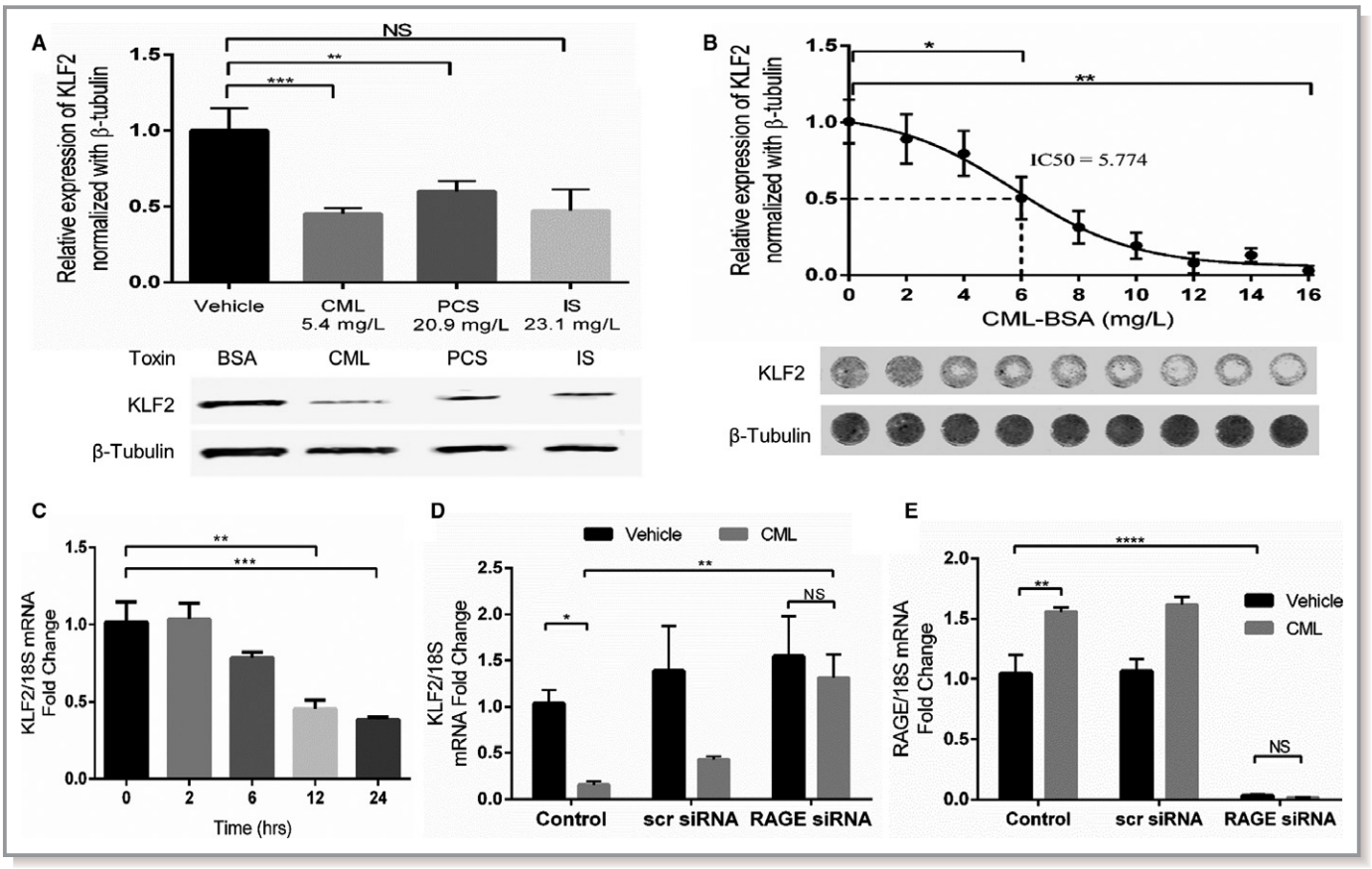

Figure 3. Uremic advanced glycation end products (AGEs) suppress endothelial Krüppel-like factor 2 (KLF2) through receptor for AGE (RAGE) signaling. A, Western blot analysis and quantification of KLF2 from human umbilical vein endothelial cells (HUVECs) exposed to individual protein-bound uremic toxins, including carboxymethyllysine (CML)-modified BSA, p-cresol sulfate (PCS), indoxyl sulfate (IS), or vehicle BSA for 24 hours (n=5 per group; 1-way ANOVA with the Dunn post hoc test; repeated 3 separate times). B, In-cell Western analysis of KLF2 expression from HUVECs treated with increasing concentrations of CML-modified BSA (n=8 per group; repeated 3 separate times). Quantification of KLF2 protein expression is relative to untreated cells and normalized to β-tubulin expression. A sigmoidal fit of the densitometry data was used to determine the IC50 of CML. C, Changes in KLF2 mRNA expression were assessed over a 24-hour time course by quantitative real-time PCR (qPCR) in HUVECs incubated for 24 hours with CML (5.4 mg/L). One-way ANOVA with the Dunn post hoc test was used to calculate statistical significance between time points (n=6 per group; repeated 3 separate times). D and E, Knockdown of RAGE expression abolished the suppression of endothelial KLF2 after CML treatment. RAGE or scrambled (scr) small interfering RNA was transfected before treatment with CML (5.4 mg/L) for 24 hours. Expression of KLF2 (D) and RAGE (E) mRNA was assessed by qPCR (n=4 per group; 2-way ANOVA with the Dunn post hoc test; repeated 3 separate times). Data are presented as mean±SEM. NS indicates nonsignificant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

in a dose-dependent decrease in the expression of KLF2 (Figure 3B), with an IC50 of 5.8 mg/L. Furthermore, time course studies (Figure 3C) conducted in HUVECs treated with CML-BSA over 24 hours showed significant decreases in KLF2 mRNA beginning at 12 hours compared with baseline (P=0.0002) and maintained after 24 hours. To assess if the effects of carboxymethyllysine-AGE were mediated by RAGE signaling, we transfected cells with RAGE small interfering RNA before AGE exposure. Knockdown of RAGE normalized KLF2 mRNA (Figure 3D) compared with cells without small interfering RNA (1.31±0.25 versus 0.18±0.04; P=0.008). We also observed that CML-BSA significantly upregulated the expression of RAGE on endothelial cells compared with vehicle BSA (Figure 3E).

AGE-Mediated Suppression of KLF2 Promotes Endothelial Dysfunction

To assess whether AGE-mediated suppression of endothelial KLF2 is associated with endothelial dysfunction, we measured oxidative stress and leukocyte adhesion in AGE-treated HUVECs after transduction of a KLF2 overexpression adenovirus (Figure 4). CML-BSA significantly increased ROS production (Figure 4A and 4C) and monocyte adhesion (Figure 4E) in HUVECs transduced with empty vector, compared with vehicle BSA. In contrast, ROS production and monocyte adhesion were significantly diminished in cells overexpressing KLF2 compared with empty vector (P<0.0001 for both). Similarly, both 5% and 10% uremic serum significantly increased ROS production (Figure 4B and 4D) and monocyte adhesion (Figure 4F) compared with equivalent concentrations of nonuremic serum. Overexpression of KLF2 also significantly decreased ROS production and monocyte adhesion in the presence of 5% and 10% uremic serum compared with empty vector (P<0.0001 for both concentrations). These results suggest that AGE-mediated suppression of KLF2 promotes oxidative stress and the expression of adhesion molecules associated with endothelial dysfunction.

Activation of Nuclear Factor-κB Signaling Mediates KLF2 Suppression

Prior studies suggest that activation of the nuclear factor-κB (NF-κB) signaling pathway is responsible for the suppression of KLF2 by proinflammatory stimuli.[22,36] Because NF-κB is also a key pathway activated by RAGE signaling,[20,37,38] we first tested whether blockage of NF-κB signaling was capable of attenuating the AGE-mediated suppression of KLF2. We transduced HUVECs with an adenovirus expressing a dominant-negative mutant of IκBα, which has serine-to-alanine substitutions at amino acids 32 and 36, which are resistant to phosphorylation-induced degradation of IκBα. Transduction of HUVECs with a dominant-negative mutant of IκBα attenuated carboxymethyllysine-mediated suppression of KLF2 compared with control vector (Figure 5A and 5B). These results were also associated with a reduction in carboxymethyllysine-mediated migration of the p65 subunit of NF-κB to the nucleus, which is responsible for inhibiting KLF2 transcription (Figure 5C). These results suggest that activation of NF-κB signaling by RAGE is responsible for suppressing endothelial KLF2.

RAGE Antagonists Inhibit Suppression of KLF2 in Vitro

Finally, we investigated if administration of novel RAGE antagonists was capable of restoring KLF2 expression and improving markers of EC function. HUVECs were treated with increasing concentrations of the RAGE antagonist azeliragon (TTP488) in the presence or absence of CML-BSA. TTP488 prevented the suppression of KLF2 mRNA by CML-BSA with an EC50 of 1.9 μmol/L compared with vehicle-treated cells (Figure 6A). At concentrations >5 μmol/L, TTP488 completely restored KLF2 expression to baseline levels. We also ascertained whether TTP488 could improve endothelial function in the presence of the complete uremic milieu (Figure 6B through 6E). In the presence of uremic serum for 24 hours, TTP488 did not increase the expression of KLF2 or its downstream targets, endothelial NO synthase and thrombomodulin, compared with cells without TTP488 (Figure 6B through 6D). However, pretreatment with the RAGE antagonist did reduce the expression of vascular cell adhesion molecule 1 by half in cells exposed to uremic serum (P=0.0068; Figure 6E).

Discussion

Patients with advanced CKD and ESRD have impaired vascular health. This results in significant morbidity and mortality attributable to CVD and dialysis vascular access dysfunction, ostensibly 2 different but linked manifestations of uremic vascular biological features. Despite the magnitude and appreciation of a potential “reverse epidemiology” in this area, there are no effective therapies for this important clinical problem,[39] perhaps because of a lack of knowledge about the specific mediators involved in this process. In this context, the data presented herein address an important knowledge gap by identifying KLF2 as a key regulator of endothelial function, which is highly repressed by the uremic milieu and protein-bound uremic solutes, such as AGEs. We were also able to demonstrate that uremic AGEs reduce the expression of KLF2 and target genes in cultured endothelial cells through NF-kB activation, resulting in impaired endothelial function.

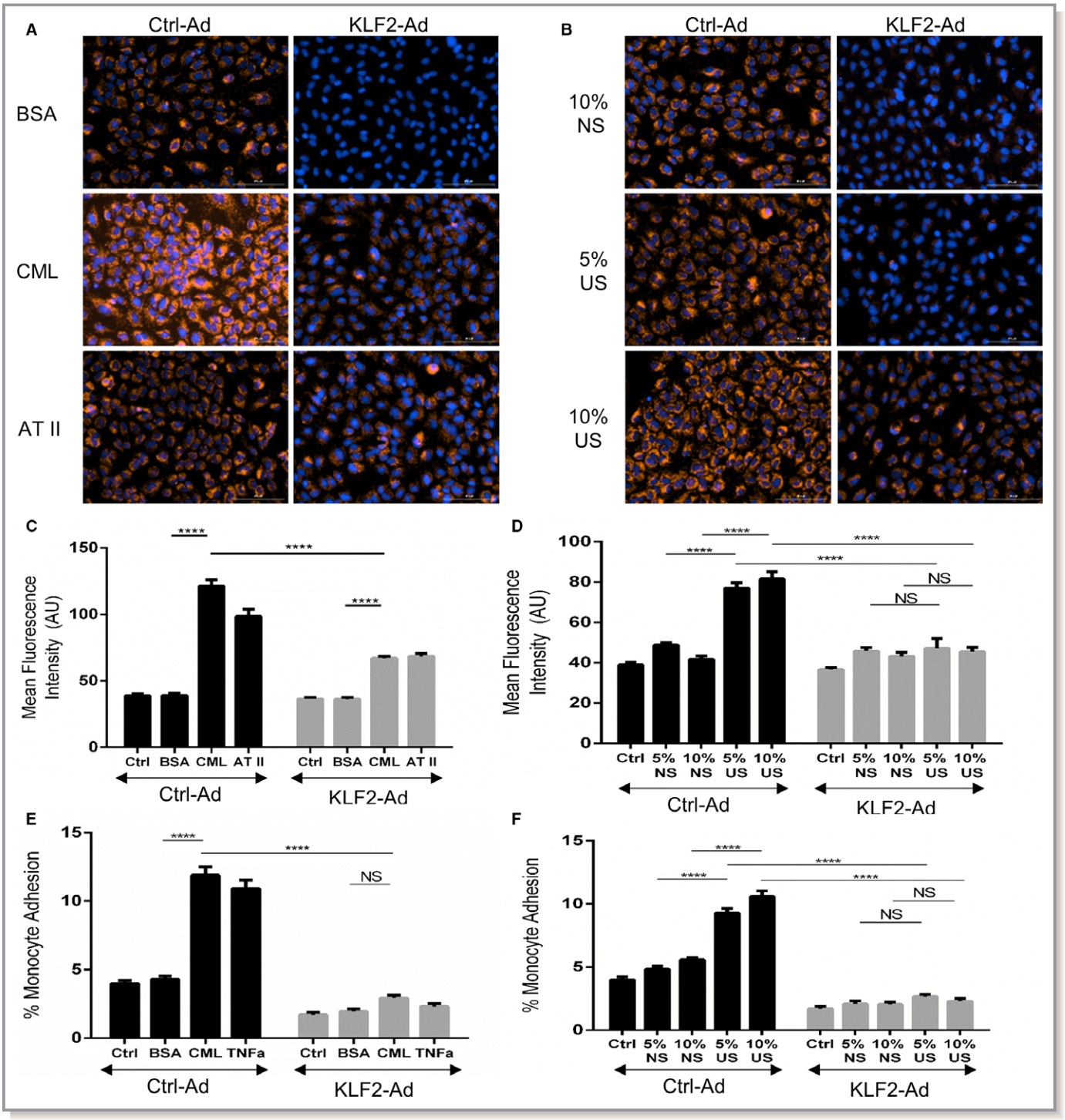

Figure 4. Uremic advanced glycation end products (AGEs) induce endothelial reactive oxygen species (ROS) and leukocyte adhesion through suppression of Krüppel-like factor 2 (KLF2). Human umbilical vein endothelial cells (HUVECs) were transfected with KLF2 overexpression adenovirus (recombinant adenovirus expressing KLF2 and green fluorescent protein [GFP] [KLF2-Ad]) or empty vector (recombinant adenovirus expressing GFP [Ctrl-Ad]) and exposed to the AGE compound carboxymethyllysine (CML)-modified BSA at 5.4 mg/L, unmodified BSA, nonuremic porcine serum (NS), or uremic porcine serum (US) for 24 hours. ROS production was measured in AGE- or serum-treated cells using the CellROX Orange Reagent fluorescent probe with 100 nmol angiotensin II (AT II) as a positive control. Representative images from AGE (A) and serum (B) treated cells are shown. C and D, Cellular ROS production was quantified by measuring individual cell fluorescence intensity in 16 high-power fields from AGE (C) or serum (D) treated cells (n=18 per group; 2-way ANOVA with the Holm-Sidak post hoc test; repeated 3 separate times). E and F, Monocyte adhesion to HUVEC monolayers was measured in AGE (E) or serum (F) treated cells using Calcein red-orange labeled THP-1 cells, with tumor necrosis factor α (TNFα) as a positive control (Ctrl; n=18 per group; 2-way ANOVA with the Holm-Sidak post hoc test; repeated 3 separate times). Data are presented as mean±SEM. AU indicates arbitrary unit; and NS, nonsignificant. ****P<0.0001.

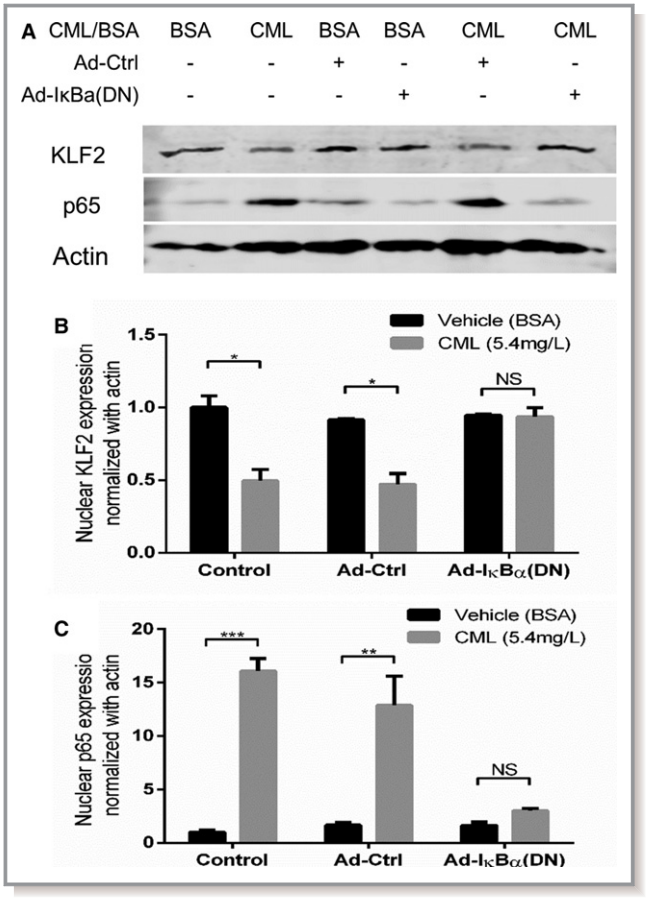

Figure 5. Uremic suppression of Krüppel-like factor 2 (KLF2) is mediated by nuclear factor-κB signaling. A, Human umbilical vein endothelial cells were pretreated for 48 hours with control adenovirus (recombinant adenovirus expressing green fluorescent protein [GFP] [Ad-Ctrl]) or adenovirus with a dominant-negative IκBα (Ad- IκBα[DN]), followed by exposure to 5.4 mg/L carboxymethyllysine-modified BSA or vehicle BSA for 12 hours. Nuclear extracts were harvested from cells, and Western blots for KLF2, the p65 subunit, and actin were performed. Representative Western blots are shown. B and C, Nuclear KLF2 and p65 were normalized to actin levels. Quantification of nuclear KLF2 and p65 relative to vehicle-treated controls and normalized to actin expression (n=3 per group; repeated 3 separate times). Two-way ANOVA with the Dunn post hoc test was used to calculate statistical significance between treatment groups. Data are presented as mean±SEM. NS indicates nonsignificant. **P*<0.05, ***P*<0.01, ****P*<0.001.

Research conducted on KLF2 and other Krüppel-like factors during the past several years has identified its role as a key "molecular switch" that regulates a large proportion of the transcriptome, thereby affecting virtually all endothelial functions.[22,40] In this study, we used uremic serum from pigs with characterized renal insufficiency to assess the impact of uremia on endothelial KLF2 and function. To our understanding, this model provided the best internal controls to study the direct effect of multiple uremic toxins (with potential exponential impact) on endothelial cells, without the confounding effects of medications, risk factors, or other disease states. With uremic porcine serum, we found that exposure of endothelial cells to even 10% uremic porcine serum suppresses the expression of KLF2 in vitro. Loss of KLF2 was also associated with increased endothelial ROS production, leukocyte adhesion, and apoptosis at increasing serum concentrations. As such, we reasoned that loss of >50% of KLF2 expression by uremia leads to endothelial cell activation and eventual death. Consistent with this hypothesis, in vivo studies using global or endothelial-specific deletion of KLF2 in mice result in death during embryonic development because of loss of vascular integrity.[23,41] However, a hemizygous deletion of KLF2 in mice is viable but accelerates CVD.[42,43] This process may also help explain the loss of endothelial progenitor cells associated with ESRD.[7,44]

Protein-bound uremic toxins comprise most molecules that are linked to endothelial dysfunction and correlate with the poor prognosis of patients with ESRD.[6,7,45] Although many of these molecules are low in molecular weight, they can be considered high-molecular-weight substances while bound to albumin (68 kDa). As a result, these molecules are generally poorly removed by hemodialysis because only the free fraction is available for diffusion through the dialysis membrane. For example, Lesaffer et al showed that the percentage removal of PCS and IS by high-flux hemodialysis was only 29% and 34%, respectively.[46] However, the use of hemodiafiltration or prolonged hemodialysis has proved to be beneficial in improving the removal of these solutes.[47] Given these links, we investigated the impact of prolonged dialysis (>24 hours) on endothelial KLF2 expression. Using serum that had undergone prolonged dialysis, we found that suppression of KLF2 was minimal compared with baseline levels, indicating that differential concentrations of uremic toxins present in the nondialyzed serum are responsible for the transient changes in KLF2 expression. This effect of prolonged hemodialysis for removing protein-bound uremic toxins was also highlighted by Cornelis et al, who showed increased reduction ratio and total solute removal of multiple protein-bound solutes if dialysis time was extended up to 8 hours.[4] However, the clinical relevance of these findings and the impact of increasing protein-bound toxin clearance on endothelial function need to be addressed in controlled, prospective, clinical trials.

We also assessed the specific impact of several protein-bound uremic toxins, including PCS, IS, and carboxymethyllysine-modified albumin. These molecules are representative of protein-bound solutes in the phenol, indole, and AGE categories, respectively, and have some of the highest in vivo concentrations compared with the normal population.[35] Our studies showed that 2 solutes, PCS and carboxymethyllysine, at concentrations commonly found in uremia, induced a suppression of endothelial KLF2. However, we cannot exclude the possibility that solutes having no effect

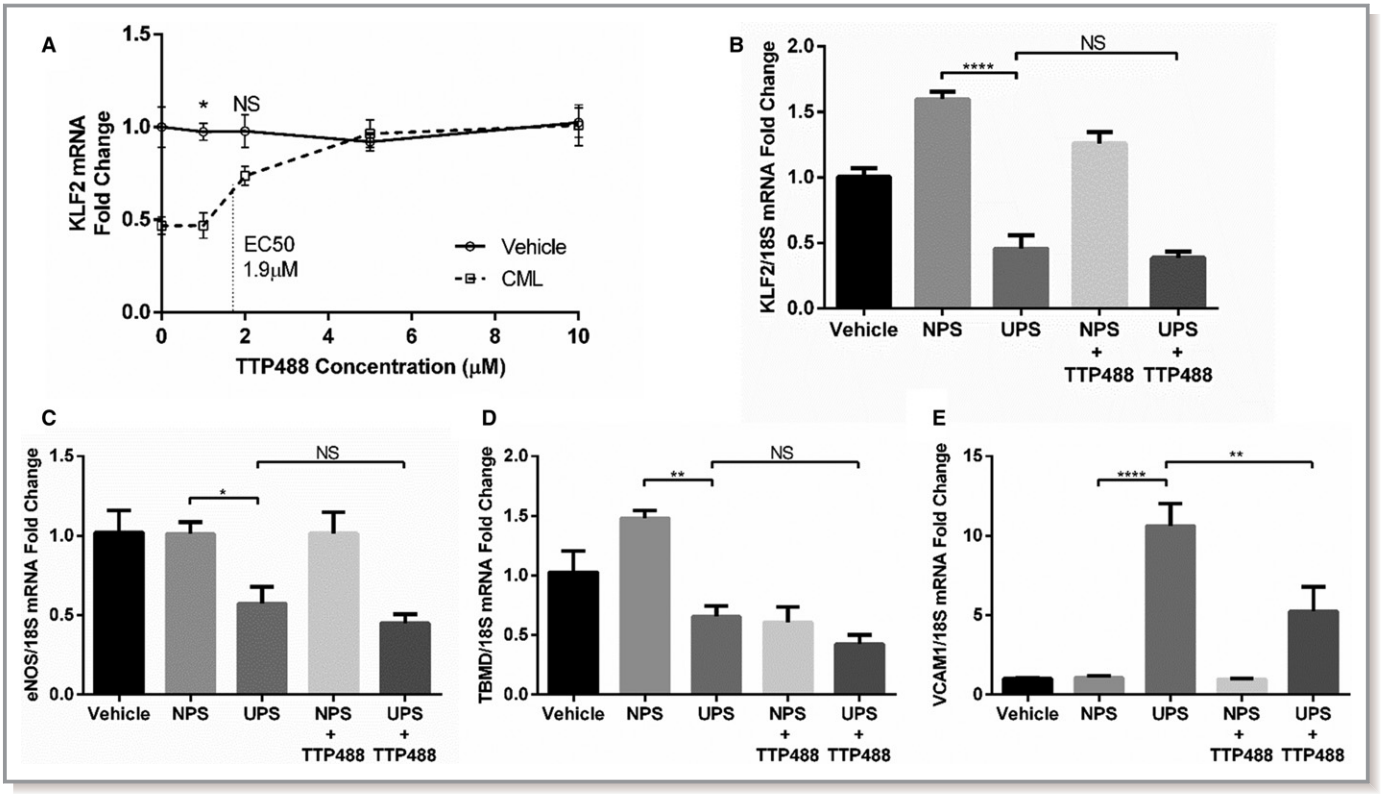

Figure 6. Uremic suppression of Krüppel-like factor 2 (KLF2) is not blocked by the receptor for advanced glycation end product (RAGE) antagonist azeliragon (TTP488). A, Human umbilical vein endothelial cells (HUVECs) were treated with 1 to 5 μmol/L of the RAGE antagonist TTP488 for 4 hours, followed by exposure to 5.4 mg/L carboxymethyllysine (CML)-modified BSA for 12 hours. KLF2 transcript levels were determined by real-time quantitative (qPCR). B through E, HUVECs were pretreated with 5 μmol/L TTP488, followed by exposure to 10% uremic (UPS) or nonuremic (NPS) porcine serum for 12 hours. Expression levels of KLF2 (B) and regulated genes, including endothelial NO synthase (eNOS; C), thrombomodulin (TBMD; D), and vascular cell adhesion molecule 1 (VCAM1; E), were assessed by qPCR (n=4 per group; repeated 3 separate times). Data are presented as mean±SEM. NS indicates nonsignificant. **P*<0.05, ***P*<0.01, *****P*<0.0001 (2-way ANOVA with the Dunn post hoc test).

in the present study may have an effect with longer incubation times or higher concentrations. Numerous in vitro and in vivo studies have implicated AGEs with endothelial dysfunction through interactions with RAGE. Engagement of RAGE by AGE ligands has been shown to inhibit endothelial NO production, increase ROS, and promote inflammation.[18,48,49] In addition, serum levels of AGE protein adducts positively correlate with in vivo tests of endothelial dysfunction and atherosclerotic disease in patients with ESRD.[50] Although these studies point towards a critical role of AGEs in the pathogenesis of endothelial dysfunction, the direct mechanistic links between AGEs and their endothelial effects have not been clearly delineated. Given that many of these processes are regulated by KLF2, our findings may provide a causal mechanism linking AGEs and the high prevalence of endothelial dysfunction in patients with kidney disease.

In addition to inflammatory mediators, KLF2 is highly regulated by hemodynamic shear stress.[23,51] These 2 stimuli function in a mutually antagonistic manner to regulate KLF2 expression, and the balance of inflammation (uremia) and shear stress may dictate the degree of endothelial dysfunction or activation.[36,51,52] Therefore, application of laminar shear stress to endothelial cells exposed to a proinflammatory uremic milieu may blunt changes in KLF2 expression. Indeed, our studies show that preconditioning of endothelial cells under arterial levels of wall shear stress blocks the uremic suppression of KLF2 in vitro. This observation is noteworthy because it suggests that uremia may have a synergistic effect in enhancing atherosclerosis and neointimal hyperplasia in regions of low or oscillatory shear stress through suppression of KLF2. Consistent with this hypothesis, studies in patients with ESRD have shown a higher degree of atherosclerotic plaque within the carotid artery bifurcation, with disturbed flow and low levels of shear stress.[53] In addition, Parmar and colleagues have implicated silencing of KLF2 with increased leukocyte adhesion and lack of protection against oxidative stress in endothelial cells preconditioned with atheroprotective flow.[54] Similarly, we also observed protection against leukocyte adhesion and ROS production in cells overexpressing KLF2, even under static flow conditions. Taken together,

this evidence suggests that therapies that promote KLF2 expression by reducing abnormal shear stress or proinflammatory mediators could be useful at preventing both CVD and dialysis vascular access dysfunction in the populations with CKD and ESRD.

In summary, we have demonstrated that uremic solutes present in serum during renal insufficiency result in the suppression of endothelial KLF2, a major regulator of endothelial homeostasis. This reduction in KLF2 is associated with increased endothelial dysfunction, particularly through the AGE pathway. We also show a link between hemodynamics, uremia, and KLF2 expression. Taken together, suppression of KLF2 constitutes an important mechanism by which uremic toxins impair endothelial function. These initial findings may result in the identification of novel therapeutic targets to improve overall vascular health in patients with CKD and ESRD.

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Disclosures

Prabir Roy-Chaudhury is a Consultant/Advisor for WL Gore, Bard Peripheral Vascular, Medtronic, Cormedix, TVA, Akebia, and Relypsa; and is the Founder/Chief Scientific Officer for Inovasc. The remaining authors have no disclosures to report.

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