Advanced glycation end products inhibit adhesion ability of differentiated podocytes in a neuropilin-1-dependent manner

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Bondeva T, Wojciech S, Wolf G. Advanced glycation end products inhibit adhesion ability of differentiated podocytes in a neuropilin-1-dependent manner. Am J Physiol Renal Physiol 301: F852–F870, 2011.—Podocyte injury can occur by a number of stimuli. Maintaining of an intact podocyte structure is essential for glomerular filtration; therefore, podocyte damage severely impairs renal function. Recently, we have reported that addition of glycated BSA [advanced glycation end products (AGE)-BSA] to differentiated murine podocytes inhibited neuropilin-1 (NRP1) expression and dramatically influenced podocyte migration ability (Bondeva T, Ruster C, Franke S, Hammerschmid E, Klagsbrun M, Cohen CD, Wolf G. Kidney Int 75: 605–616, 2009; Bondeva T, Wolf G. Am J Nephrol 30: 336–345, 2009). The present study analyzes the influence of AGEs and NRP1 on podocyte adhesion and cytoskeleton reorganization. We show that treatment with AGE-BSA significantly reduced podocyte adhesion to collagen IV, laminin, and fibronectin compared with Co-BSA (nonglycated BSA)-incubated cells, which was further augmented by transient inhibition of NRP1 expression using NRP1 short interference (si) RNA. On the other hand, forced overexpression of NRP1 markedly increased the adhesion ability of podocytes to the ECMs despite the AGE-BSA treatment. No changes were observed when podocyte adhesion to collagen I was assayed. These findings were also manifested with disorganization of podocyte actin stress fibers and decreased lamellipodia formation processes due to AGE-BSA treatment or NRP1 suppression. In addition, AGE-BSA or suppression of NRP1 both reduced the phosphorylation of focal adhesion kinase (FAK) and Erk1/2 in PMA-stimulated differentiated podocytes. Analysis of RhoA family GTPase activity demonstrated that treatment with AGE-BSA or NRP1 depletion inhibited as well the activation of the Rac-1 and Cdc42 and did not affect RhoA activity. All these effects were reversed by forced overexpression of full-length NRP1 cloned into the pcDNA3 vector in differentiated podocytes. Our study demonstrates that AGEs, in part via suppression of NRP1 expression, decreased podocyte adhesion and contribute to reduction of Rac-1 and Cdc42 GTPase activity. These effects may be further responsible for the podocytes damage and loss in diabetic nephropathy. Our findings suggest a role for NRP1 in regulating the podocyte actin cytoskeleton, and therefore reduction of NRP1 expression could be critical for podocyte function.

Age-related diabetes; diabetic nephropathy; focal adhesion kinase; neuropilin-1; podocytes; diabetics; advanced glycation end products; glycated BSA; neuropilin-1; inhibition; adhesion; cytoskeleton; AGE-BSA; NRP1; FAK; Erk1/2; RhoA family GTPase; Rac-1; Cdc42; Podocyte adhesion; AGE formation; diabetes mellitus; nondiabetic rats; albuminuria; histological changes; cell migration; adhesion; oxidative stress; sRAGE; RAGE; RAGE interaction; diaphanous-1; pathological changes; diabetic nephropathy; AGEs; semaphorin-3A; neuropilin-1; vascular development; class 3 semaphorins; neuronal guidance; angiogenesis; VEGF; VEGF-A; chemo repulsion; podocyte migration; kidney epithelial cells; ezrin-dependent manner.

Previously, we found that application of AGE-BSA (glycated endotoxin-free BSA) in cultured immortalized mouse differentiated podocytes reduced the expression of neuropilin-1 (NRP1) compared with Co-BSA (control, nonglycated BSA) and this effect was associated with a decrease in podocyte migration (5, 6). Inhibition of NRP1 expression with short interference (si) RNA in podocytes demonstrated the same effect (5). NRP1 is a coreceptor for class 3 semaphorins in neuronal guidance and for the angiogenic cytokine VEGF or VEGF-A in vascular development (14, 25, 33). NRP1 has a large extracellular domain and a short cytoplasmic tail and forms a complex with a plexin-A1-signaling receptor to transduce semaphorin-3A signals in neuronal cells, implicated in chemo repulsion and neuronal cell migration (15, 24, 42–44). In endothelial cells, NRP1 binds VEGF and enhances VEGFR-2-dependent VEGF functions, including cell migration and angiogenesis (23–25). In addition, NRP1 expression levels were found to be upregulated in the murine model of dermal wound healing (28). Although cell adhesion is one of the main functions of NRP1 in other cellular systems, in podocytes it has so far received little attention. Moreover, the potential molecular mechanisms whereby AGEs may regulate podocyte adhesion in diabetic nephropathy are incompletely understood. However, sequestration of AGEs with prolonged sRAGE application improved renal function and also reduced albuminuria in db/db mice (52). Significant reduction of the

Address for reprint requests and other correspondence: G. Wolf, Klinik für Innere Medizin III, Friedrich-Schiller-Univ., Erlanger-Allee 101, D-07740 Jena, Germany (e-mail: Gunter.Wolf@med.uni-jena.de).
podocyte number per glomerulus and podocyte damage occur early in experimental models of diabetic nephropathy as well as in human diabetic nephropathy (16, 53). In this study, we investigated whether AGEs and NRP1 may contribute to changes in podocyte adhesion and cytoskeleton reorganization using immortalized murine podocytes as a model system. Our data show that treatment of podocytes with AGE-BSA or downregulation of NRP1 both reduced podocyte adhesion to collagen IV, laminin, and fibronectin but did not affect podocyte adhesion to collagen I. Analysis of the proteins involved in the regulation of the cytoskeleton reorganization demonstrated that AGE-BSA inhibited focal adhesion kinase (FAK) and Erk1/2 phosphorylation. In agreement with this, we detected reduced amounts of the GTP-bound form of the small Rho GTPase Rac-1 but not of RhoA. Similar effects were observed in podocytes in which NRP1 expression was attenuated. On the other hand, forced overexpression of murine NRP1 was able to overcome AGE-BSA dependent inhibition of FAK and Erk1/2. It also rescued activation of the small GTPase Rac-1. Our data provide clear evidence of a direct effect of AGE-BSA on podocyte adhesion and cytoskeleton organization through a disruption of the functional NRP1 receptor complex, an event associated with inhibition of FAK and Rac-1 activation.

**MATERIALS AND METHODS**

**Cell culture.** Conditionally immortalized mouse podocytes (generous gift of Dr. Peter Mundel, University of Miami, Miami, FL) were grown in RPMI 1640 medium with stable glutamine (PromoCell, Heidelberg, Germany) supplemented with 10% heat-inactivated FBS (PAN Biotech, Aidenbach, Germany) in the presence of recombinant mouse γ-interferon (10 U/ml) for 3 wk at 33°C and 5% CO2 (31). Removal of γ-interferon and a temperature switch to 37°C induced podocyte differentiation. Differentiated podocytes were tested positive for synaptopodin by immunohistochemistry (IHC). These differentiated podocytes, called podocytes from now on, were used for all experiments and were grown in RPMI 1640 medium containing 10% heat-inactivated FBS at 37°C and 5% CO2. All media were supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin.

**Preparation of AGE-BSA.** AGE-BSA preparation was performed as described in our prior studies (9, 37). Briefly, BSA (fraction V, fatty acid poor, endotoxin free, Calbiochem, La Jolla, CA) was incubated under sterile conditions at 37°C for 50 days in PBS with or without the addition of glucose (90 mg/ml). After that, the fractions were filtrated (Millipore Labscale TFF System, Billerica, MA) and lyophilized. Generated glycation products in AGE-BSA and control BSA without glucose addition (Co-BSA) samples were analyzed as previously described (9, 37). For assaying the effect of AGE-BSA or Co-BSA on differentiated murine podocytes usually cells were starved for 24 h in RPMI 1640 medium containing 0.1% heat-inactivated FBS, followed by exposure to RPMI 1640 medium supplemented with 0.1% FBS plus Co-BSA (5 mg/ml) or AGE-BSA.
with 0.1% FBS plus AGE-BSA (5 mg/ml) for an additional 24 h and then subjected to the corresponding analysis.

**Downregulation of NRP1 expression in differentiated podocytes.** Podocytes were plated in 6-cm dishes at a density of $1 \times 10^6$ cells/plate in 6 ml RPMI medium containing 8% FBS. The next day, the cells were transiently transfected using HiPerfect transfection reagent (Qiagen, Hilden, Germany) with primer duplexes targeting mouse NRP1 (Mm_NRP1_2 HP siRNA) and a control, nonsilencing primer (both purchased from Qiagen). Transfections were performed as previously described (5). After that, the podocytes were subjected to adhesion assays or IHC analysis as indicated. In parallel, down-regulation of NRP1 was tested via Western blotting.

**Transient overexpression of full-length murine NRP1 in podocytes.** Podocytes were plated at a density $2 \times 10^6$ cells/plate in 10-cm plates and transfected the next day using Lipofectamine Plus transfection reagents (Invitrogen, Karlsruhe, Germany). Usually, 8 μg of the plasmid expressing full-length mouse NRP1 cloned into pcDNA3 under the control of a CMV promoter (5) or an empty pcDNA3 vector per plate were transfected in RPMI serum-free medium for 6 h. After that, the transfection medium was exchanged with a complete culture medium according to the manufacturer’s instructions. Transfected podocytes were further treated as necessary and subjected to adhesion assays, Western blot analysis, or IHC staining.

**Western blot analysis.** After treatments were performed, podocytes were washed with PBS and lysed in Complete-M lysis buffer (Roche, Mannheim, Germany) containing a protease inhibitor cocktail (Roche) and 100 μM Na$_2$VO$_4$ (Sigma, Taufkirchen, Germany). The protein content was determined, and amounts equal to 20–30 μg/lane were separated on 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane by a semidry Western blotting transfer cell (Bio-Rad Laboratories, Hercules, CA) as described elsewhere (6). The following antibodies were used to assess protein expression: anti-NRP1 rabbit polyclonal antibody from Santa Cruz Biotechnology (Heidelberg, Germany), anti-vinculin monoclonal antibody from Sigma, anti-phospho-Erk1/2 monoclonal antibody from Cell Signaling Technology (Karlsruhe, Germany), anti-Erk 2 monoclonal antibody, anti-phospho-FAK Tyr-576 rabbit polyclonal antibody, and anti-FAK mouse monoclonal antibody from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated secondary antibodies were from KPL (Gaithersburg, MD). The proteins were visualized using ECL detection reagent (Roth, Karlsruhe, Germany) and LAS 3000 digital software (Fujifilm Life Science, Santa Clara, CA). Mouse VEGF164 and PMA used for podocyte stimulation were from Sigma. The phosphorylation of the Erk1/2 or FAK was analyzed using Image J 1.44 software or MultiGauge (Fujifilm Life Science) software and normalized to the protein expression of the total Erk1/2 or FAK protein. The data are expressed as fold relative to corresponding control treatment in each experiment. The same method was used to normalize for protein expression or Rac-1 activity.

**Cell adhesion assay.** Collagen IV-, laminin-, collagen I-, and fibronectin-coated 24-well plates were used to test differentiated podocyte adhesion ability. Collagen IV, laminin, and fibronectin were purchased from Millipore (Schwalbach, Germany), collagen I was purchased from Sigma. Briefly, the plates were incubated with 200 μl of 10 μg/ml collagen IV, 10 μg/ml collagen I, or 10 μg/ml fibronectin, or 20 μg/ml laminin for 1 h at 37°C. After that, the excess solution was removed, and the plates in the hood were air dried. The plates were further used for the adhesion assay experiments. Differentiated podocytes were grown in complete culture medium followed by serum deprivation for 24 h. After that, the cells were grown for an additional 24 h in RPMI 1640 supplemented with 5 mg/ml Co-BSA or 5 mg/ml AGE-BSA. Next, the cells were detached using acutase (PAA Laboratories, Cölbe, Germany), and $1 \times 10^5$ podocytes were allowed to attach to ECM protein-coated plates in 300 μl medium containing 0.1% FBS in RPMI 1640 medium. Time course adhesion assays were performed for 30-min, 1-h, and 3-h periods at 37°C in a

![Fig. 2. A and B: wound-healing assay. Podocytes were grown to achieve a confluent monolayer. After that, the cells were serum-deprived for 8 h and a scratch was made. Scratches were measured at 0 and 24 h. A: representative images of the cell wound closure after 24 h are shown for podocytes grown in the presence of Co-BSA (left) and AGE-BSA (right). B: quantification of podocyte wound closure mediated by Co-BSA and AGE-BSA. Bars show the percentage of the wound closure after 24 h relative to 0 h. In the presence of AGE-BSA, podocyte wound closure was significantly attenuated (*$P < 0.05$ compared with Co-BSA, $n = 3$).](Image)
5% CO₂ incubator. After that, the nonadherent cells were washed thoroughly with PBS and the plates were incubated with 200 µl/well acutase for 10 min at 37°C to detach the adherent cells. The cell number was counted with the Easy cell counter (Roche Innovatis, Bielefeld, Germany). For adhesion assay experiments with transfected podocytes, the cells were transiently transfected for 24 h with the corresponding plasmids or siRNA duplexes. Afterward, the cells were serum-deprived for 24 h followed by an adhesion assay with the corresponding ECMs for 30 min. In addition, time course adhesion assays were performed for podocytes transfected with NRP1 full-length plasmid or siRNA duplexes together with the corresponding control transfections. Twenty-four-hour posttransfection podocytes were treated with Co-BSA or AGE-BSA for an additional 24 h, followed by adhesion assays as indicated in figure legends. Each experiment was performed with at least four replicates.

**Pull-down assays.** The activation of Rac-1 or Cdc42 was measured in a pull-down assay using GST-CRIB, a glutathione S-transferase (GST)-tagged Cdc42 and Rac-1-interacting domain of p21-activated kinase, which binds to the active or GTP-bound form of Cdc42 or Rac-1 (23). Purified GST-CRIB protein was purchased from Jena Bioscience (Jena, Germany). The activation of RhoA was assayed in a pull-down assay using GST-rotekin as bait. Purified GST-rotekin was obtained from Jena Bioscience. Briefly, 3 × 10⁵ cells, treated as shown in figure legends, were washed twice with ice-cold PBS and lysed in 600 µl Complete Lysis-M buffer (Roche), containing an inhibitor cocktail (Roche) and 100 µM Na₃VO₄. Protein concentrations were determined, and equal amounts of protein were allowed to bind to 2 µg of purified GST-CRIB or GST-rotekin proteins for 2 h at 4°C. After that, to each pull-down assay 40 µl of GST beads (50% slurry) were added for 1 h at 4°C. Beads were collected by centrifugation at 3,000 rpm for 5 min and washed three times with lysis buffer, containing the inhibitor cocktail. The bound protein complexes were eluted with 40 µl 2× Laemmli buffer and analyzed for bound GTP-Rac-1 using anti-Rac-1 antibody (Santa Cruz Biotechnology), for GTP-Cdc42 using anti-Cdc42 antibody (Cell Signaling Technology, Karlsruhe, Germany), or for GTP-RhoA using anti-RhoA antibody (Sigma) by Western blotting. The amounts of Rac-1, Cdc42, or Rho A in the protein lysates used for the pull-down experiments were also tested by Western blotting.

**Wound-healing assay in vitro.** Differentiated podocytes were grown in six-well plates until a confluent cell monolayer was achieved. The cells were serum-starved in RPMI 1640 medium supplemented with 0.1% FBS for 8 h, and then a scratch wound was created using a 1- to 10-µl pipette tip. Podocytes were then cultured in RPMI 1640 medium with 0.1% FBS supplemented with 5 mg/ml Co-BSA or 5 mg/ml AGE-BSA for an additional 24 h. To determine the rate of motility, each cell monolayer was wounded at 0 and 24 h using Axiomat software (Zeiss, Jena, Germany) and an Axiovert 25 Zeiss microscope (Zeiss). An antibody against FAK (Santa Cruz Biotechnology) or for GTP-RhoA using anti-RhoA antibody (Sigma) by Western blotting. The amounts of Rac-1, Cdc42, or Rho A in the protein lysates used for the pull-down experiments were also tested by Western blotting.

**IHC.** For IHC, podocytes were plated on poly-1-lysine-coated chamber slides. The cells were treated with Co-BSA or AGE-BSA or transfected as needed. Afterward, the podocytes were fixed in 4% paraformaldehyde for 20 min at room temperature. The primary antibodies were used at a dilution of 1:100 and incubated overnight at 4°C, and the secondary antibodies, goat anti-rabbit Alexa 488-conjugated or goat anti-rabbit Cy3-conjugated, both from Invitrogen, were used at a dilution of 1:500 for 3 h. The cells were then washed several times with PBS, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and the cells were embedded in Kaiser’s gelatin (Merck, Darmstadt, Germany). Staining was detected by fluorescent microscopy using Axiomat software (Zeiss). An antibody against phosphorylated Tyr-567 was used for detection of phosphorylated FAK (Santa Cruz Biotechnology). For detection of filamentous actin (F-actin), TRITC-labeled phalloidin (Sigma) or Alexa 488-conjugated phallolidin (Invitrogen) was used according to the manufacturer’s recommendations.

**Quantification of cellular extensions.** The lengths of the cellular extensions of the podocytes were quantified using Image J 1.44 software in combination with a Wacom pen tablet (Wacom). The cellular extensions were defined as projections initiating from the cell body including the membrane protrusions. The length of cellular extensions was determined, and equal amounts of protein were allowed to bind to 2 µg of purified GST-CRIB or GST-rotekin proteins for 2 h at 4°C. After that, to each pull-down assay 40 µl of GST beads (50% slurry) were added for 1 h at 4°C. Beads were collected by centrifugation at 3,000 rpm for 5 min and washed three times with lysis buffer, containing the inhibitor cocktail. The bound protein complexes were eluted with 40 µl 2× Laemmli buffer and analyzed for bound GTP-Rac-1 using anti-Rac-1 antibody (Santa Cruz Biotechnology), for GTP-Cdc42 using anti-Cdc42 antibody (Cell Signaling Technology, Karlsruhe, Germany), or for GTP-RhoA using anti-RhoA antibody (Sigma) by Western blotting. The amounts of Rac-1, Cdc42, or Rho A in the protein lysates used for the pull-down experiments were also tested by Western blotting.

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extensions in Co-BSA-treated cells or Co-BSA- and PMA-stimulated cells was set as 100%. The number of the lamellipodia formation per cell was also counted. In addition, the length of actin fibers was determined after IHC staining as described above using the pen tablet.

Statistical analysis. All data are reported as means ± SD. Statistical analysis was performed using the statistical package SPSS for Windows version 11.0 (SPSS, Chicago, IL). Results were analyzed with the Kruskal-Wallis test followed by the Mann-Whitney U-test. Differences were considered significant when \( P < 0.05 \).

**RESULTS**

AGE-BSA treatment reduced differentiated podocyte adhesion ability. Recently, we have shown that AGE-BSA exposure significantly inhibited NRP1 expression in differentiated murine podocytes (5). We also found that both AGE-BSA treatment and suppression of NRP1 expression using NRP1 siRNA impaired podocyte migration in vitro in a Transwell migration

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**Fig. 4.** A–D: effect of AGE-BSA on Coll IV and laminin adhesion in podocytes with manipulated expression of NRP1 via NRP1 siRNA transfection or transient overexpression of exogenous full-length murine NRP1. A and B: differentiated murine podocytes were transfected with siRNA duplexes for NRP1 or co siRNA. Twenty-four hours posttransfection, cells were treated with 5 mg/ml Co-BSA or 5 mg/ml AGE-BSA and podocyte adhesion ability was analyzed in a time-dependent manner on Coll IV- and laminin-coated plates. In all experiments, the number of adherent cells detected in Co-BSA-treated co siRNA-transfected podocytes set as 100%. A: adhesion to Coll IV of podocytes depleted of NRP1 expression. Adhesion of the podocytes was assayed for 30-min, 1-h, and 3-h time periods. Treatment with AGE-BSA significantly reduced adhesion compared with Co-BSA \((^* P < 0.05\) vs. Co-BSA, \(n = 3\) ). Downregulation of NRP1 significantly further impaired podocyte adhesion to Coll IV compared with the co siRNA-transfected podocytes under the same experimental conditions. All experiments were performed 3 times, each with 4 replicates, \((^* P < 0.05, ^{#} P < 0.001, n = 3)\). B: podocytes were transfected and treated as in A, and the adhesion to laminin was analyzed for 30-min \((n = 3)\), 1-h \((n = 3)\), and 3-h \((n = 3)\) time periods. Treatment with AGE-BSA significantly reduced adhesion compared with Co-BSA \((^+ P < 0.05, ^{++} P < 0.001\) vs. Co-BSA, \(n = 3\) ). Podocytes depleted of NRP1 protein demonstrated further reduced adhesion ability to laminin compared with the co siRNA-transfected podocytes. The adhesion in podocytes treated with AGE-BSA was significantly inhibited relative to co siRNA at 30 min and 3 h, but was not further able to inhibit podocytes adhesion at the 1-h time point (nsd). The experiments were performed at least 3 times, each with 4 replicates. \(#P < 0.001\). C: adhesion to Coll IV in podocytes transfected with empty vector or full-length NRP1. Transient overexpression of NRP1 significantly induced podocyte adhesion compared with the empty vector-transfected podocytes in Co-BSA- as well as in AGE-BSA-treated podocytes. The differences between Co-BSA- or AGE-BSA-treated podocytes overexpressing NRP1 was not significant. \((^{**} P < 0.001, n = 3)\).
assay. Therefore, we further analyzed the AGE-BSA and the NRP1 contribution to differentiated murine podocyte adhesion in vitro conditions. Podocyte adhesion ability was assayed as a time course adhesion assay for 30 min-, 1-h, and 3-h periods using different ECM proteins as collagen IV, laminin, fibronectin, and collagen I. Fibronectin, collagen IV, and laminin are all present in the glomerular basement membrane (GBM) (32), whereas collagen I is a typical constituent of a provisional angiogenic ECM (19, 40). Adhesion assays were performed with differentiated murine podocytes. We found that 5 mg/ml AGE-BSA not only significantly decreased the short-term adhesion of podocytes to collagen IV ($P < 0.001$ vs. Co-BSA, $n = 8$) but also reduced podocyte adhesion ability after 1 h ($P < 0.01$, $n = 3$) and 3 h ($P < 0.03$, $n = 3$) compared with the Co-BSA-treated cells (Fig. 1A). A similar effect was observed in laminin adhesion assays. AGE-BSA exposure inhibited the short-term adhesion to laminin ($P < 0.01$ vs. Co-BSA, $n = 6$) as well as in the 1-h ($P < 0.01$, $n = 6$) and 3-h adhesion test ($P < 0.005$, $n = 3$, see Fig. 1B). Interestingly, whereas the tendency of podocyte adhesion to collagen IV slightly improved with time from 50.48 to 61.46%, we found that podocyte adhesion to laminin was further reduced with time (Fig. 1B). We even followed podocyte adhesion in laminin-coated plates after 6 h and found that it was even more suppressed (data not shown). Next, we tested the adhesion of podocytes to fibronectin. Our data show that attachment of the

Fig. 5. A–F: AGE-BSA treatment reduces PMA-dependent Erk1/2 and focal adhesion kinase (FAK) phosphorylation in differentiated podocytes. A: protein lysates from podocytes left untreated or stimulated with 20 ng/ml VEGF164, $10^{-6}$ M PMA, or $10^{-7}$ M PMA were subjected to Western blot analysis of p-Erk1/2. Addition of PMA but not VEGF164 induced Erk1/2 activation in podocytes (top). Equal protein loading was detected using Erk2 monoclonal antibody (bottom). Representative Western blots are shown ($n = 3$). B: detection of NRP1 expression in protein lysates from differentiated podocytes treated with 0.1% FBS, 5 mg/ml Co-BSA, or 5 mg/ml AGE-BSA for 24 h followed by stimulation with $10^{-7}$ M PMA for 10 min. Equal loading was controlled by detection of vinculin expression. ($n = 5$). C: detection of Erk1/2 phosphorylation in protein lysates from differentiated podocytes treated as in B. Equal loading was analyzed via detection of total Erk2 protein levels. Representative Western blots are shown ($n = 3$ independent experiments with qualitatively similar results). D: relative p-Erk1/2 expression. p-Erk1/2 expression was normalized to total Erk2 expression. p-Erk1/2 levels in control (nontreated) cells incubated with 0.1% FBS were set as 1. AGE-BSA significantly reduced p-Erk1/2 levels in PMA-treated differentiated podocytes ($*P < 0.05$ vs. PMA+AGE-BSA, $n = 3$). E: effect of AGE-BSA on FAK Tyr-576 phosphorylation (p-FAK). Cells were treated as in B, and protein lysates were tested for FAK Tyr-576 phosphorylation by Western blotting using p-FAK-specific antibodies. Equal protein loading was analyzed via detection of the total FAK protein amount in the whole cell lysates. Representative Western blots are shown. F: relative p-FAK expression. p-FAK expression was normalized to total FAK expression. p-FAK levels in control (nontreated) cells incubated with 0.1% FBS were set as 1. AGE-BSA significantly reduced pFAK levels in PMA-treated differentiated podocytes ($#P < 0.05$ vs. PMA+AGE-BSA, $n = 3$).
podocytes to fibronectin was inhibited by AGE-BSA by ~32% ($P < 0.01, n = 8$) compared with Co-BSA (Fig. 1C). Adhesion to fibronectin after 1 h did not change significantly, but after 3 h incubation of the cells on fibronectin-coated plates the cell number of the attached cells was further reduced ($P < 0.01$ vs. Co-BSA, $n = 3$). Collagen I adhesion capacity of the AGE-BSA-pretreated podocytes did not changed significantly after 30 min or 1-h time points relative to Co-BSA-pretreated podocytes but showed a significant difference after 3-h incubation on collagen I-coated plates ($P < 0.01$ vs. Co-BSA, $n = 4$, Fig. 1D). In addition, we also studied podocyte adhesion in in vitro wound healing. Scratches were allowed to close in the presence of Co-BSA (5 mg/ml) or AGE-BSA (5 mg/ml). Analysis after 24 h revealed that the distance between leading cells was larger in the presence of AGE-BSA compared with Co-BSA (Fig. 2, A and B).

NRP1 promotes differentiated podocyte adhesion. We further analyzed whether diminished podocyte adhesion due to prolonged AGE-BSA exposure is related to reduced NR1P expression by investigating the influence of NR1P on podocyte adhesion to ECM proteins. In particular, we depleted differentiated podocytes from NR1P by transient transfection of siRNA, targeting mouse NR1P expression. Podocytes were transiently transfected with NR1P siRNA or control nonsilencing siRNA and then were subjected to an adhesion assay as described above. NR1P downregulation was controlled by Western blotting. Short-term adhesion showed that loss of NR1P greatly impaired podocyte adhesion to collagen IV ($P < 0.05$ vs. control siRNA, $n = 5$) (Fig. 3A) and fibronectin ($P < 0.05$ vs. control siRNA, $n = 5$, Fig. 3A) but did not affect adhesion to collagen I (Fig. 3A). Next, we analyzed the effect of mouse NR1P transient overexpression (5) on podocyte ECM-induced adhesion. Compared with the empty vector-transfected cells, forced NR1P overexpression significantly promoted podocyte adhesion to collagen IV ($P < 0.02$ vs. empty vector, $n = 8$, Fig. 3B) and fibronectin ($P < 0.05$ vs. empty vector, $n = 8$, Fig. 3B). These results suggest that podocyte adhesion is dependent on intact NR1P expression. We also analyzed more detailed time course adhesion of podocytes transfected with full-length NR1P or NR1P siRNA on collagen IV- and laminin-coated plates when the cells were treated in addition with Co-BSA or AGE-BSA for 24 h. We found that NR1P downregulation reduced podocyte adhesion to collagen IV in Co-BSA-treated cells after 30 min ($P < 0.001$ relative to the control siRNA, $n = 3$, Fig. 4A), and upon AGE-BSA treatment podocyte adhesion was further suppressed ($P < 0.001$ relative to control siRNA-transfected cells, $n = 3$). This effect was also observed after 1- and 3-h adhesion...
assays (Fig. 4A). Analysis of the time-dependent laminin adhesion have shown that inhibition of NRP1 was able to decrease short-term podocyte attachment to laminin in Co-BSA-treated cells ($P < 0.05$ vs. control siRNA Co-BSA, $n = 3$, Fig. 4B) but to a lesser extend compared with collagen IV adhesion. It also further augmented the negative effect of AGE-BSA on podocytes adhesion to ($P < 0.05$ vs. control siRNA AGE-BSA, $n = 3$), whereas AGE-BSA alone reduced the adhesion to $60.2 \pm 8.5\%$, $n = 3$, $P < 0.03$ vs. Co-BSA control siRNA, $n = 3$ (Fig. 4B). The one-hour adhesion period on laminin-coated plates demonstrated that NRP1 depletion attenuated podocyte adhesion ($P < 0.03$ vs. Co-BSA control siRNA, $n = 3$) but was unable to further reduce the adhesion abnormality caused by AGE-BSA alone in control siRNA-transfected cells (Fig. 4B). The prolonged adhesion time period up to 3 h demonstrated that, similarly to the 30-min short-term adhesion, NRP1 downregulation was effectively suppressing podocyte adhesion to laminin in Co-BSA- as well as AGE-BSA-treated cells, correspondingly ($P < 0.05$ vs. control siRNA Co-BSA, $n = 3$ and $P < 0.05$ vs. control siRNA Co-BSA, $n = 3$, Fig. 4B). The negative influence on podocyte adhesion due to AGE-BSA treatment was reversed by transient

Fig. 7. A–F: influence of NRP1 overexpression on Erk1/2 and FAK activation (phosphorylation) in podocytes. Cells were transiently transfected with an empty vector (pcDNA3) or full-length mouse NRP1 cloned into pcDNA3 (NRP1). Twenty-four hours posttransfection, cells were serum-starved and left untreated or stimulated with PMA. NRP1 expression and phosphorylation state of Erk1/2 and FAK were detected with Western blotting. The blots were stripped and reprobed for the corresponding loading controls. A: expression of NRP1 in transiently transfected podocytes. Representative Western blots demonstrate NRP1 overexpression in podocytes. β-Actin expression shows equal loading; $n = 3$. B: graphic presentation of densitometry results of NRP1 expression normalized to β-actin protein expression. There was a significant increase in NRP1 in podocytes transfected with the overexpression construct ($P < 0.01$, $n = 3$). C: influence of NRP1 upregulation on Erk1/2 phosphorylation. Total Erk2 expression was used as a loading control. Representative Western blots of $n = 3$ independent experiments are shown. D: relative p-Erk1/2 levels where p-Erk1/2 expression was normalized to total Erk2 expression. p-Erk1/2 levels in cells transfected with empty vector under control conditions, incubated with 0.1% FBS, were set as 1. NRP1 overexpression significantly increased pErk1/2 levels in PMA-treated differentiated podocytes. (*$P < 0.05$, #$P < 0.03$, $n = 3$). E: NRP1 overexpression increased FAK activation via FAK Tyr-576 phosphorylation. Representative Western blots of p-FAK and total FAK protein levels are shown ($n = 3$). F: relative p-FAK levels. p-FAK expression was normalized to total FAK expression. p-FAK levels from empty vector-transfected podocytes under control conditions, incubated with 0.1% FBS, were considered as 1. Transfection of full-length mouse NRP1 significantly induced pFAK phosphorylation in PMA-treated differentiated podocytes. It significantly increased as well the basal p-FAK levels in the absence of PMA (*$P < 0.05$, #$P < 0.03$, $n = 3$).
overexpression of full-length murine NRP1 on collagen IV as well as laminin-coated plates (Fig. 4, C and D). Time course collagen IV adhesion experiments showed that after 30 min podocyte adhesion was increased \( (P < 0.001 \text{ vs. empty vector Co-BSA- or AGE-BSA-treated cells}) \), \( P < 0.001 \text{ vs. empty vector, } n = 3 \), (Fig. 4C). Time course adhesion assays performed on laminin-coated plates revealed that transient overexpression of NRP1 enhanced podocyte adhesion compared with empty vector-transfected and AGE-BSA-treated cells (Fig. 4D).

Unfortunately, we could not analyze the effect of NRP1 downregulation on podocytes in the in vitro wound-scratching assay as we were unable to maintain siRNA knockdown in transiently transfected podocytes long enough to achieve the confluent cell monolayer necessary to efficiently perform these studies.

**AGE-BSA treatment impaired adhesion signaling in podocytes.** The observation that podocytes exposed to AGE-BSA or after depletion of NRP1 were characterized by reduced adhesion ability suggests the possibility that these treatments induced impaired adhesion signal transduction. Activation of focal adhesion kinase (FAK) and Erk are well known regulators of cell adhesion processes (1, 2, 10, 11, 20, 21). Therefore, we first analyzed the activation of Erk1/2 in podocytes by two agonists: mouse VEGF (VEGF164) and PMA by Western blotting (29, 30, 34). Erk1/2 is known as a downstream target of VEGF and PMA stimulation (36, 43). We found that 20 ng/ml VEGF164 (a rather high concentration) was unable to induce Erk1/2 activation in differentiated podocytes (Fig. 5A, lanes 2 and 3). We also used lower concentrations (5 and 10 ng/ml VEGF164) or higher concentrations (of up to 50 ng/ml VEGF164), but we were not able to observe phosphorylation

![Fig. 8. A–F: impact of NRP1 depletion on FAK and Erk1/2 activation in podocytes pretreated with Co-BSA or AGE-BSA. Podocytes transfected with control siRNA or NRP1 siRNA duplexes 24 h after transfections were incubated with Co-BSA or AGE-BSA for an additional 24 h, followed by 10−7 M PMA stimulation for 10 min where indicated. After that, the cells were lysed and the extracted proteins were subjected to Western blot analysis. A: suppression of NRP1 expression via AGE-BSA or NRP1 siRNA transfection. Representative Western blots of NRP1 expression and equal protein loading detected via vinculin expression are shown (n = 3). B: relative NRP1 expression in protein lysates from Co siRNA- and NRP1 siRNA-transfected podocytes normalized to vinculin expression. AGE-BSA treatment significantly reduced NRP1 expression compared with Co-BSA-treated cells (+P < 0.05, n = 3). NRP1 expression in NRP1-depleted podocytes treated with Co-BSA was significantly lower relative to co siRNA Co-BSA (+P < 0.05 vs. Co siRNA) and AGE-BSA (+P < 0.05 vs. co siRNA) treatment. NRP1 protein levels detected in lysates from Co-BSA-treated (co siRNA) samples were set as 1 (n = 3). C: NRP1 suppression further inhibits PMA-dependent Erk1/2 phosphorylation relative to Co-BSA or AGE-BSA. Representative Western blots of p-Erk1/2 and total Erk2 protein expression, used as a loading control, are shown (n = 3). D: relative p-Erk1/2 levels where p-Erk1/2 expression was normalized to total Erk2 expression. p-Erk1/2 levels from co siRNA-transfected cells incubated with Co-BSA without PMA were set as 1. AGE-BSA in the presence of PMA treatment of podocytes significantly reduced p-Erk1/2 expression (+P < 0.05 vs. Co-BSA + PMA, n = 3). NRP1 depletion significantly reduced p-Erk1/2 levels in PMA-treated differentiated podocytes in the presence of Co-BSA (+P < 0.05) or AGE-BSA (+P < 0.05) relative to co siRNA (n = 3). E: NRP1 suppression significantly inhibits FAK Tyr-576 phosphorylation in lysates from Co-BSA-treated cells. Representative Western blots of p-FAK and total FAK protein expression, used as a loading control, are shown (n = 3). F: relative p-FAK levels where p-FAK expression was normalized to total FAK expression. p-FAK levels from co siRNA-transfected cells under Co-BSA treatment (nonstimulated with PMA) were set as 1. AGE-BSA + PMA treatment of podocytes significantly reduced p-FAK expression (+P < 0.05 vs. Co-BSA + PMA, n = 3). NRP1 depletion abolished PMA-dependent FAK phosphorylation in Co-BSA-treated differentiated podocytes (+P < 0.05 vs. co siRNA, n = 3) but could not further reduce p-FAK expression in AGE-BSA-treated cells.

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of Erk1/2 by Western blotting (data not shown). However, we detected a strong Erk1/2 activation when podocytes were treated for 10 min with $10^{-7}$ M (Fig. 5A, lanes 4 and 5) or $10^{-6}$ M PMA (Fig. 5A, lanes 6 and 7). In agreement with our previous findings, NRP1 expression did not differ in Co-BSA- or 0.1% FBS-treated podocytes, while AGE-BSA reduced its expression (Fig. 5B) (5, 6). Furthermore, treatment with PMA for 10 min did not increase NRP1 expression in AGE-BSA-treated cells (Fig. 5B). As both PMA concentrations were equally potent in inducing Erk1/2 activation, we used the lower PMA concentration for additional experiments. We also tested whether AGE-BSA treatment could suppress PMA-induced Erk1/2 activation. We found no significant difference between whether AGE-BSA treatment could suppress PMA-induced Erk1/2 activation. We used the lower PMA concentration for additional experiments. We also tested whether AGE-BSA treatment could suppress PMA-induced Erk1/2 activation. We found no significant difference between whether AGE-BSA treatment could suppress PMA-induced Erk1/2 phosphorylation (Fig. 5, C and D). Next, we assayed the activation state of FAK by Western blot analysis of FAK phosphorylation at Tyr-576, as FAK Tyr 576/577 phosphorylation by Src kinase increases the activity of FAK and creates binding sites for other ligand effectors (10, 11). As shown in Fig. 5, E and F, p-FAK levels in response to PMA were strongly inhibited when podocytes were preincubated with AGE-BSA ($P < 0.05$ vs. Co-BSA, $n = 3$), whereas differences between 0.1% FBS and Co-BSA samples stimulated with PMA were not statistically significant (Fig. 5F). Therefore, we demonstrated that prolonged AGE-BSA treatment reduced the levels of p-FAK and p-Erk1/2 in podocytes.

**NRP1 suppression impaired adhesion signaling in podocytes.** Next, we tested whether podocyte depletion of NRP1 using NRP1 siRNA would affect Erk1/2 and FAK activities. Western blot analysis revealed that Nrp siRNA transfection specifically reduced NRP1 expression (Fig. 6, A and B) but did not affect total vinculin, Erk 2, or FAK protein expression (Fig. 6, A, C, D).
and E). We also found that NRP1 suppression reduced PMA-dependent Erk1/2 phosphorylation (P < 0.03 vs. control siRNA, n = 3) (Fig. 6, C and D). Similarly, reduction of NRP1 expression attenuated FAK Tyr-576 phosphorylation by PMA stimulation compared with control siRNA-transfected cells treated with PMA (P < 0.01 vs. control siRNA, n = 3, Fig. 6, E and F). We also observed that these p-FAK levels were lower than the basal p-FAK levels. On the other hand, forced NRP1 overexpression in podocytes (Fig. 7, A and B) further increased PMA-induced Erk1/2 and FAK phosphorylation relative to the empty vector-transfected cells. Western blot analysis show that both p-Erk1/2 (P < 0.03 vs. empty vector, n = 3, Fig. 7, C and D) and p-FAK Tyr-576 (P < 0.03 vs. empty vector, n = 3, Fig. 7, E and F) levels were strongly increased by NRP1 overexpression. In addition, NRP1 transfection significantly increased as well the basal p-FAK levels (P < 0.05 vs. empty vector, n = 3, Fig. 7, E and F). Thus we clearly demonstrated that NRP1 downregulation in differentiated podocytes was sufficient to maintain the negative regulation on Erk1/2 and FAK signaling.

NRP1 overexpression rescued inhibitory effect of AGEs on adhesion signaling in differentiated podocytes. We further analyzed by Western blotting the effect of NRP1 downregulation or forced overexpression on Erk1/2 and FAK phosphorylation in podocytes treated with Co-BSA or AGE-BSA for 24 h before PMA stimulation. We found that transient depletion of

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Fig. 10. A–D: AGE-BSA treatment induced cytoskeleton reorganization and changed FAK distribution in differentiated murine podocytes. A and B: differentiated podocytes grown on chamber slides were Co-BSA or AGE-BSA treated for 24 h. After that, in some slides the cells were stimulated with 10^{-7} M PMA for 10 min. Then, the cells were fixed, permeabilized, and filamentous actin (F-actin) was visualized using TRITC-labeled phalloidin (red channel), or the podocytes were stained with an antibody specific for phosphorylated FAK at Tyr-576 (p-FAK) followed by Alexa 488-conjugated secondary antibody (green channel). Nuclei were visualized using 4'-6-diamidino-2-phenylindole (DAPI) staining (blue channel). The cells were analyzed using fluorescent microscopy. Representative images from 3 independent experiments are shown. Bar = 10 μm. A: detection of F-actin and p-FAK staining in podocytes treated with Co-BSA alone or in the presence of PMA. Arrows in F-actin staining demonstrate the membrane protrusion and cytoskeleton reorganization induced by PMA stimulation. Arrow in pFAK staining shows the localization of pFAK at adhesion points. B: detection of F-actin and pFAK staining in podocytes treated with AGE-BSA alone or in the presence of PMA. F-actin stress fibers are very thin and dispersed compared with Co-BSA treatment. It is also obvious that the podocytes treated with AGE-BSA did not respond to the PMA stimulus and have mostly cytosolic p-FAK staining. F-actin, ×3 zoom image. C: quantification of F-actin fiber length. The average length of F-actin fibers measured in Co-BSA-treated cells was set as 100%. Treatment with AGE-BSA significantly decreased the length of the F-actin fibers (*P < 0.001 vs. Co-BSA). For each treatment, the length of F-actin fibers from 20 cells was measured. D: number of lamellipodia cell processes per cell in Co-BSA- and AGE-BSA-treated podocytes stimulated with PMA. Addition of AGE-BSA to the differentiated podocytes resulted in a significant reduction of the lamellipodia formation processes (*P < 0.001 vs. Co-BSA). Lamellipodia were counted from at least 20 cells.
NR1P with NR1P1 siRNA further inhibited the NR1P1 expression in AGE-BSA-treated cells (Fig. 8, A and B). This effect was associated with a stronger inhibition of the phosphorylated Erk1/2 levels compared with AGE-BSA-treated podocytes (Fig. 8, C and D) and completely blocked the FAK phosphorylation as observed by Western blotting (Fig. 8, E and F). We further evaluated the effect of transient overexpression of full-length NR1P in podocytes treated with Co-BSA or AGE-BSA. We found that this resulted in significantly elevated phosphorylation of Erk1/2 and FAK despite AGE-BSA treatment of the podocytes (Fig. 9, C–F). We also observed that NR1P protein levels were increased at least twice in lysates from AGE-BSA-treated podocytes relative to the empty vector AGE-BSA lysates (Fig. 9, A and B).

**AGE-BSA treatment affects podocyte cytoskeleton rearrangement.** Cell migration and adhesion are processes regulated by protein complexes inducing cytoskeleton rearrangement. The observed reduction in podocyte migration and adhesion abilities as well as the inhibition of Erk1/2 and FAK activity after treatment with AGE-BSA or suppression of mouse NR1P1 expression using NR1P1 siRNA suggest the hypothesis that these events are connected to the rearrangement of the podocyte cytoskeleton. Therefore, we investigated whether AGE-BSA and NR1P1 mediate reorganization of F-actin in differentiated podocytes. Cells were grown on chamber slides and incubated with Co-BSA or AGE-BSA for 24 h. F-actin was visualized via staining with TRITC-labeled phalloidin, and the slides were analyzed using fluorescence microscopy. We observed that AGE-BSA treatment induced changes in podocyte F-actin organization compared with the Co-BSA-treated cells. Whereas in cells incubated with Co-BSA the F-actin fibers were long and well structured (Fig. 10A), AGE-BSA treatment led to a dispersed structure of F-actin with very thin filaments (arrows, Fig. 10B; F-actin staining). In addition, PMA treatment induced large, membrane protrusions (lamellipodia) in the presence of Co-BSA (arrows, Fig. 10A; F-actin staining) which were absent in AGE-BSA treated cells (Fig. 10B; F-actin). Moreover, using the same experimental conditions, we analyzed the distribution and localization of the phosphorylated FAK at Tyr 576 (p-FAK) by IHC. As seen in Fig. 10A (p-FAK staining), in the absence of PMA Co-BSA-treated podocytes show p-FAK staining characterized by an accumulation of the signal at the edge of the podocyte extensions (arrow). In contrast, AGE-BSA treatment induced changes in pFAK cellular distribution compared with Co-BSA, p-FAK signals were weaker at the cell membrane and mainly accumulated in the cytoplasm (Fig. 10B; p-FAK staining). The p-FAK staining was mostly membrane localized, around the cell adhesion points, in Co-BSA-treated podocytes stimulated with PMA (Fig. 10A; p-FAK), whereas in PMA-stimulated podocytes treated with AGE-BSA p-FAK signals were mostly cytosolic and membrane localization was difficult to detect (Fig. 10B; p-FAK). Another important observation was that AGE-BSA-treated cells appeared to be retracted from the surface (Fig. 10B; F-actin, AGE-BSA plus PMA, arrows). We also found that the length of the actin fibers in AGE-BSA-treated podocytes was significantly shorter compared with Co-BSA-treated cells, $P < 0.01$ vs. Co-BSA (Fig. 10C), and there were few lamellipodia cell processes formed in AGE-BSA and PMA stimulation, whereas in Co-BSA- and PMA-treated cells we observed up to 12 lamellipodia formations (Fig. 10D).

**NR1P suppression affects podocyte cytoskeleton rearrangement.** Next, we studied the influence of NR1P1 downregulation via transient siRNA transfection of control siRNA and NR1P1 siRNA and analyzed F-actin cytoskeleton organization and p-FAK distribution in differentiated podocytes. While F-actin

![Fig. 11. A and B: NR1P1 suppression induced cytoskeleton reorganization and changed FAK distribution in differentiated podocytes. Podocytes were transiently transfected with control siRNA or NR1P1 siRNA duplexes for 24 h. Then, the cells were serum-deprived for 24 h and left untreated (0.1% FBS) or stimulated with 10^{-7} M PMA for 10 min. F-actin and p-FAK nuclei were visualized using DAPI staining (blue channel). Representative images from 3 independent experiments are shown. Cell staining was analyzed using a fluorescent microscope. Bar = 10 μm. A: detection of F-actin and p-FAK in co siRNA-transfected podocytes treated with 0.1% FBS and PMA. B: detection of F-actin and p-FAK in NR1P1 siRNA-transfected podocytes treated with 0.1% FBS and PMA. Transient suppression of NR1P1 impaired the F-actin organization in differentiated podocytes as well as p-FAK cellular distribution. Similar to the AGE-BSA-treated podocytes (Fig. 6B), the p-FAK staining is mostly cytosolic in PMA-treated podocytes, and membrane proliferations and philopodia are not formed.](http://ajprenal.physiology.org/)
and p-FAK staining did not differ significantly between control siRNA-transfected (Fig. 11A) and Co-BSA-treated podocytes (Fig. 10A), depletion of NRP1 demonstrated similarity in F-actin and p-FAK staining to that observed in the AGE-BSA-exposed cells (Fig. 10B). Suppression of NRP1 was characterized by dispersed, disorganized F-actin fibers, and PMA stimulation did not induce formation of lamellipodia processes (Fig. 11B; F-actin staining) as in the Co-BSA-treated podocytes (Fig. 10A; F-actin) or control siRNA-transfected cells (Fig. 11A; F-actin). Staining of p-FAK was also decreased compared with control siRNA (Fig. 11B; p-FAK) and, similarly to AGE-BSA, we detected mostly cytosolic membrane localization of p-FAK staining when cells were stimulated with PMA. While NRP1 suppression prevented the formation of lamellipodia in podocytes after the addition of PMA (Fig. 11B), transfection of control siRNA did not (Fig. 11A; F-actin). We also believe that the p-FAK staining detected in AGE-BSA- and NRP1 siRNA-transfected podocytes stimulated with PMA correlates with the levels of the p-FAK observed from protein lysates by Western blotting. This demonstrates that NRP1 suppression contributes to the observed changes in F-actin organization in AGE-BSA-treated podocytes.

**Overexpression of NRP1 rescued the negative effect of AGE-BSA on podocyte cytoskeleton reorganization.** Next, we analyzed the effect of NRP1 overexpression on podocyte cytoskeleton reorganization and p-FAK distribution. The cytoskeleton actin organization and pFAK localization in podocytes transfected with an empty vector did not differ from that already demonstrated in Fig. 10A (data not shown) or control siRNA transfection presented in Fig. 11A. We observed that the podocytes transfected with NRP1 pcDNA3 were somewhat

A

![F-actin and p-FAK localization in podocytes transfected with an empty vector, Co-BSA, and AGE-BSA](http://ajprenal.physiology.org/)

B

![F-actin and p-FAK localization in podocytes transfected with NRP1 pcDNA3, Co-BSA, and AGE-BSA](http://ajprenal.physiology.org/)
larger and were characterized with a development of a numerous extensions in the Co-BSA- as well as in AGE-BSA-treated podocytes (Fig. 12A; F-actin staining). The cells look “more differentiated,” and each cell developed more primary and secondary extensions (arrows, Fig. 12A). The addition of PMA promoted a strong response and severely disturbed the formation of lamellipodia and membrane protrusions in every extension even in the small one (arrows, Fig. 12B; F-actin). These effects were also seen when the podocytes overexpressing NRP1 were treated with AGE-BSA alone or stimulated with PMA. As shown in Fig. 12B, NRP1 was able to prevent AGE-BSA-dependent suppression of PMA stimulation, and the podocytes responded to the PMA stimulus with a formation of lamellipodia and show typical membrane p-FAK staining and distribution of the pFAK all over the lamellipodia processes (arrows, Fig. 12B; p-FAK staining).

**AGE-BSA and NRP1 suppression inhibits activation of small GTPases Rac-1 and Cdc42 but not of RhoA.** We further assayed the activity of Rho family GTPases Rac-1, Cdc42 and RhoA using a pull-down assay where GST-CRIB and GST-rotekin were correspondingly used as bait to bind the activated GTP-loaded Rac-1 and Cdc42 or GTP-loaded RhoA proteins. We found that AGE-BSA treatment reduced the basal levels of GTP-bound Rac-1, and PMA stimulation was not effective in inducing further Rac-1 activation (Fig. 13, A and B). In addition, we tested the influence of NRP1 siRNA on small GTPase activation. Western blot analysis of the corresponding pull-down assays showed that suppression of NRP1 completely abolished Rac-1 activation under PMA stimulation (Figs. 13, C and D). We routinely assayed as well the expression of total Rac-1 protein in the cell lysates used for the pull-down assays. We did not find any differences in total

![Figure 13](http://ajprenal.physiology.org/)

**Fig. 13.** A–F: AGE-BSA and NRP1 depletion suppressed Rac-1 activation in podocytes. A: podocytes were treated with Co-BSA or AGE-BSA for 24 h, followed by PMA stimulation as indicated. Subsequently, cells were lysed and activated Rac-1 was pulled down using glutathione S-transferase (GST)-CRIB protein as bait. GST-Rac-1 was detected by Western blotting using GST-Rac-1 antibody after the pull-down assays. The membrane was stripped and reprobed for GST-CRIB using anti-GST antibody to detect the amount of the bound GST-CRIB as an equal loading control for the assay. Total Rac-1 was also detected in cells lysates before the pull-down experiments. Representative Western blots are shown (n = 3). B: relative GTP-Rac-1 levels normalized to GST-CRIB. The levels of GTP-bound Rac-1 from cells treated with Co-BSA only were set as 1. AGE-BSA significantly reduced the levels of GTP-Rac-1 in PMA-treated differentiated podocytes (*P < 0.05 + P < 0.03, n = 3). C: podocytes were transfected with co siRNA or NRP1 siRNA duplexes and 24 h after transfections were serum-starved followed by 10⁻⁷ M PMA stimulation. Rac-1 activation was detected on a pull-down assay as in A using the corresponding protein lysates. The amounts of the GST-CRIB protein used for the pull-down assays and the protein level of Rac-1 in the protein lysates are shown. Representative Western blots from 3 identical experiments are shown. D: relative GTP-Rac-1 levels normalized to GST-CRIB. The levels of GTP-bound Rac-1 from cells transfected with co siRNA under control conditions (non-PMA) were set as 1. NRP1 suppression significantly reduced the levels of GTP-Rac-1 (*P < 0.05, #P < 0.01, n = 3). E: podocytes transfected with an empty vector or full-length mouse NRP1 cloned into pcDNA 3 (NRP1) 24 h posttransfection were serum-deprived followed by 10⁻⁷ M PMA stimulation. Rac-1 activation was detected in a pull-down assay as in A using lysates from empty vector- and NRP1-transfected podocytes. Corresponding GST-CRIB and Rac-1 loading controls are presented (n = 3). F: relative GTP-Rac-1 levels normalized to GST-CRIB. The levels of GTP-bound Rac-1 from cells transfected with an empty vector under control conditions (nontreated with PMA) were set as 1. NRP1 overexpression significantly increased the levels of GTP-Rac-1 (*P < 0.05, + P < 0.03, n = 3).
Rac-1 expression during AGE-BSA treatment (Fig. 13A; Rac-1 total lysate). We detected reduced Rac-1 expression in NRPI-depleted podocytes treated with PMA, but we did not observe such a reduction in the control (0.1% FBS)-treated cells transfected with NRPI siRNA (Fig. 13C; Rac-1 total lysate). We also tested the amounts of GST-CRIB by Western blotting. Figure 13B, middle, shows that the amount of GST-CRIB protein used in lysates depleted from NRPI stimulated with PMA is even larger than in the other samples; nevertheless, activated Rac-1 was not detected. Next, we evaluated the amounts of the GTP-bound Rac-1 when NRPI or empty vector was overexpressed in differentiated podocytes. In contrast to NRPI-depleted podocytes, NRPI overexpression increased the level of GTP-bound Rac-1 under PMA stimulation relative to the empty vector-transfected podocytes (Fig. 13, E and F). There was as well no detectable difference in total Rac-1 protein expression in NRPI-transfected podocytes (Fig. 13E; Rac-1 total lysate). Identical results we found when Cdc42 activation was analyzed. AGE-BSA and NRPI downregulation inhibited the amount of GTP-Cdc42 in PMA-stimulated podocytes compared with the cells treated with PMA in the presence of Co-BSA. We also tested whether AGE-BSA and NRPI affect GTP-bound RhoA levels. Therefore, we assayed the GTP-RhoA bound to GST-rhotekin (GST-RBD) from protein lysates of podocytes treated as for the Rac-1 activity assays (Fig. 14). We analyzed as well the levels of GTP-bound RhoA levels from lysates of podocytes transfected with NRPI siRNA or the control siRNA duplexes (Fig. 15A). Neither of the assays was able to demonstrate differences in the GTP-RhoA amount in the pull-down assays. PMA treatment also did not alter the GTP-RhoA amount detected by Western blotting from pull-down analyses (Fig. 15, A and B). We further investigated whether NRPI overexpression would be able to induce differences in the amount of GTP-RhoA bound to GST-rhotekin. The results demonstrated that neither the empty vector nor the forced NRPI overexpression was able to induce differences in the amount of RhoA detected after the pull-down assays. We found a sustained RhoA activation in differentiated podocytes (Fig. 15C). Additionally, we analyzed Rac-1 activity in podocytes transfected with NRPI siRNA or overexpressing NRPI before incubation with Co-BSA or AGE-BSA followed by PMA stimulation in some samples. We found that suppression of NRPI reduced the GTP-bound Rac-1 levels in Co-BSA- as well as in AGE-BSA-treated podocytes (Fig. 16A, Rac-1 pull-down assay). In contrast, NRPI transient transfection was able to increase the GTP-bound Rac-1 levels despite AGE-BSA treatment and also enhanced basal Rac-1 activity (Fig. 16B; Rac-1 pull-down assay).

**DISCUSSION**

The accumulation of AGEs is an aging-related process and is formed via nonenzymatic glycation of proteins. Elevated levels of AGEs are found in serum samples from diabetic patients (9, 46, 47, 55). It is also recognized that AGEs contribute to diabetic complications, including diabetic nephropathy (for a review, see Ref. 46). The important role of AGEs in the development of diabetic nephropathy has been demonstrated by studies in diabetic db/db mice in which the scavenging of AGEs with sRAGE attenuated diabetic nephropathy (52). Different AGEs are recognized by RAGE, which initiates various signaling pathways, resulting in enhanced oxidative stress and transcriptional activation. Differentiated podocytes expressed the RAGE receptor and are therefore a likely target of the AGEs-dependent effects (52). Podocytes have well-structured and highly organized foot processes and a complicated regulation of the actin cytoskeleton framework (31). Effacement of the podocytes foot processes is associated with proteinuria (32) and declining renal function. We have recently shown that treatment of podocytes with AGE-BSA, but not with Co-BSA significantly inhibited NRPI expression as well as podocyte migration (5, 6). A similar effect has been observed in podocytes with suppressed NRPI expression. Although the NRPI function in regulating cell migration and adhesion has been well studied in neuronal and vascular...
systems (22, 24, 25, 35), NRPI function in podocytes has so far received little attention. In this study, we further investigate the contribution of AGE-BSA and NRPI downregulation to podocyte adhesion and cytoskeleton organization. Podocytes are firmly attached to the GBM. Therefore, we tested the influence of AGE-BSA on podocyte adhesion to ECM components such as collagen IV, laminin, and fibronectin, which are present in the GBM (32). We found that podocyte adhesion to collagen IV-, laminin-, and fibronectin-coated plates was significantly reduced when cells were treated with AGE-BSA compared with Co-BSA. In contrast, podocyte adhesion to collagen I was independent of AGE-BSA. Similarly to the effects of AGE-BSA treatment, downregulation of NRPI expression also inhibited podocyte adhesion to collagen IV, laminin, and fibronectin but failed to affect the adhesion to collagen I. Therefore, the alteration in podocyte adhesion by NRPI is not a general, e.g., toxic, phenomenon but rather a function related to specific ECM proteins present in the GBM such as collagen IV and laminin. We also observed that downregulation of NRPI using siRNA technology was less effective in suppressing podocyte adhesion to laminin compared with collagen IV. On the other hand, NRPI overexpression significantly augmented podocyte adhesion to collagen IV, laminin, and fibronectin over the vector-transfected cells and rescued the negative effect of AGE-BSA on podocyte adhesion observed using different ECM components. These results demonstrate that AGE-BSA inhibition of podocyte adhesion is closely related to NRPI suppression. The present data correlate well with a recent finding that NRPI increases endothelial cell adhesion to fibronectin but not to collagen I (48). The authors demonstrated that this effect was independent of NRPI ligands semaphorin 3A (Sema3A) and VEGF165, which are well-known NRPI receptor ligands in neurons and endothelial cells (48). NRPI, through its cytoplasmic tail, promoted fibronectin adhesion via interaction with GAIP-interacting protein C terminus, member-1 and activation of αβ3-integrins (41). Involvement of NRPI cytoplasmic as well as extracellular domains in cell adhesion have been described (12, 26, 27, 35, 41, 48). We recently reported that NRPI expression was inhibited with diabetic nephropathy (5). Therefore, our findings could be of pathophysiological relevance. Accumulation of AGE-BSA with subsequent NRPI downregulation in diabetes likely contributes to podocyte foot process effacement and concomitant proteinuria. Podocyte primary and secondary foot processes are important characteristics of the healthy podocyte related to the reorganization of the actin stress fibers (31). Our data suggest that AGE-BSA through NRPI suppression indeed induces changes in podocyte F-actin stress fibers compared with Co-BSA- or control siRNA-transfected cells. We detected...
were stimulated as in mNRP1 or an empty vector treated with Co-BSA and AGE-BSA. The cells AGE-BSA treatment was associated with suppression of the FAK phosphorylation in podocytes. Furthermore, whereas demonstrated an inhibitory effect of AGE-BSA on Erk1/2 and cytoplasm. In addition, our analysis of adhesion signaling as the signal was observed as having accumulated mostly in the typical membrane complexes involved in the translocation of kinase after PMA stimulation, demonstrating that probably the actin fibers of the differentiated podocyte were disorganized (for a review, see Ref. 53) that antiproteinuric effects of Sema3A in mice leads to proteinuria and reduction of VEGFR2, nephrin, podocin, and CD2-associated protein expression in podocytes (45). Interestingly, we failed to induce VEGF164-dependent Erk1/2 activation in our immortalized differentiated podocytes, and they did not respond to VEGF stimulation. We also did not observe VEGFR2 expression via Western blotting in mouse podocytes (not shown) but found a low level of VEGFR2 mRNA, which was 1,000-fold lower than the NRP1 mRNA (5). Therefore, we are convinced that the observed changes in the differentiated podocytes are due to AGE-BSA treatment and NRP1 depletion and are mostly independent of VEGFR2. We also addressed the influence of AGE-BSA on the expression of diaphanous-1 (dia-1) or nephrin, as their functions in the regulation of cytoskeleton reorganization have been demonstrated (18, 22, 56). Nevertheless, we did not see any differences in the mRNA expression of dia-1 or protein expression of nephrin in AGE-BSA-treated podocytes (data not shown).

Considered in the context of the role of NRP-1 in migration and adhesion in other cellular systems and the integral relationship of actin dynamics and junction formation, our observations establish a function for NRP1 and AGEs in regulating actin cytoskeleton dynamics in podocytes via still unclear mechanisms involving FAK activation, and dependent on small GTPases Rac-1 and Cdc42 but independent of RhoA. Further studies are needed to decipher the exact NRP1-signaling complex in podocytes, but special attention should be given to the possible interplay between Rac-1 and NRP1 in regulation and stabilization of the podocyte cytoskeleton. However, manipulation of NRP1 signaling may offer a novel therapeutic approach to prevent podocyte damage by maintaining an intact cytoskeleton. In this regard, it has been recently demonstrated (for a review, see Ref. 53) that antiproteinuric effects of cyclosporin A result from the stabilization of the actin cyto-

Fig. 16. A and B: overexpression of NRP1 rescued Rac-1 activity in AGE-BSA-treated differentiated podocytes. A: podocytes were transiently transfected with co siRNA and NRP1 siRNA before treatment with Co-BSA or AGE-BSA for 24 h, followed by PMA stimulation as indicated. The activated GTP-Rac-1 was detected by Rac-1 Western blotting after the pull-down assays (top). The membrane was stripped and reprobed for GST-CRIB using anti-GST antibody to detect the amount of the bound GST-CRIB (middle). Total Rac-1 was also detected in cellular lysates used for the pull-down experiments (bottom). Downregulation of NRP1 inhibited Rac-1 activity in Co-BSA- as well as in AGE-BSA-treated podocytes stimulated with PMA compared with the co siRNA-transfected podocytes. Representative Western blots are shown (n = 3). B: detection of Rac-1 activity in podocytes transfected with full-length mNRP1 or an empty vector treated with Co-BSA and AGE-BSA. The cells were stimulated as in A, and Rac-1 activity was assayed as described above. The amounts of the activated GTP-Rac-1, bound to the GST-CRIB protein, were detected via Rac-1 Western blotting (top). The GST-CRIB protein used for the pull-down assays was detected via GST-antibody (middle). The protein level of Rac-1 in total lysates used for the pull-down experiments is also shown (bottom). Overexpression of NRP1 enhanced the basal activity of Rac-1 as well as the Rac-1 activity in Co-BSA- or AGE-BSA-treated podocytes stimulated with AGE-BSA. The inhibitory effect of AGE-BSA on Rac-1 activity was reversed by NRP1 overexpression. Representative Western blots are shown (n = 3).
sketch in podocytes via blocking the calcineurin-mediated dephosphorylation of synaptopodin.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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