Mechanical strain increases SPARC levels in podocytes: implications for glomerulosclerosis

Raghu V. Durvasula and Stuart J. Shankland

Department of Medicine, Division of Nephrology, University of Washington School of Medicine, Seattle, Washington

Submitted 2 November 2004; accepted in final form 1 May 2005

Durvasula, Raghu V., and Stuart J. Shankland. Mechanical strain increases SPARC levels in podocytes: implications for glomerulosclerosis. Am J Physiol Renal Physiol 289: F577–F584, 2005; doi:10.1152/ajprenal.00393.2004.—Glomerular capillary hypertension is a final common pathway to glomerulosclerosis. Because podocyte loss is an early event in the development of glomerulosclerosis, it is logical that the deleterious effects of glomerular capillary hypertension involve podocyte injury. Yet, the mechanisms by which elevated intraglomerular pressure is translated into a maladaptive podocyte response remain poorly understood. Secreted protein acidic and rich in cysteine (SPARC) is a matricellular protein activated in various disease states of the podocyte and accelerates renal injury, as evidenced by the milder course of experimental diabetic nephropathy in SPARC-null mice compared with diabetic SPARC wild-type mice. Accordingly, we tested the hypothesis that mechanical strain activates SPARC in podocytes and thus is a putative mediator of podocyte injury in states of intraglomerular capillary hypertension. Conditionally immortalized mouse podocytes were subjected to 10% cyclical stretch while nonstretched cells served as controls. SPARC levels were measured in whole cell lysate and cell media. Immunostaining was performed for SPARC in an experimental model of glomerular capillary hypertension. Our results demonstrate cyclical stretch of podocytes markedly increased SPARC levels in cell lysate, through activation of p38, as well as secreted SPARC. Relevance was shown by demonstrating increased podocyte staining for SPARC in the uninephrectomized spontaneously hypertensive rat. In conclusion, we have made the novel observation that mechanical forces characteristic of states of glomerular capillary hypertension lead to increased levels of SPARC in podocytes. We speculate that the increase in SPARC may be maladaptive and lead to a progressive reduction in podocyte number, thus fueling the future development of glomerulosclerosis.

Bacterial infection in the kidney, Pathogenesis of Tubulointerstitial fibrosis

METHODS

Cell culture. Experiments were performed using early passage growth-restricted, conditionally immortalized mouse podocytes (gift from Dr. R. V. Durvasula, Division of Nephrology, Box 356521, Univ. of Washington School of Medicine, Seattle, WA 98195). These cells were predominantly functional as determined by the ability to grow in 10% FBS. Cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin, and cultured in a humidified incubator at 37°C with 5% CO2. Confocal microscopy was performed using a Nikon DIAPHOT with a 40x objective lens. Images were captured using Nikon C-HR6000D digital camera and processed with Adobe Photoshop CS3 software. All images were captured with a Nikon C-HR6000D digital camera and processed with Adobe Photoshop CS3 software.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

GLOMERULAR CAPILLARY HYPERTENSION characterizes many forms of chronic kidney disease and is a final common pathway to glomerulosclerosis. Beyond primary glomerular disease, any state of reduced functional nephron mass results in elevated intraglomerular pressure (7). Pharmacological and nonpharmacological interventions that lower glomerular capillary pressure slow the rate of progression of experimental and clinical renal failure (8, 24, 25, 27). Although the cellular mechanisms by which glomerular capillary hypertension accelerates renal disease have not been entirely elucidated, studies have shown that podocyte injury plays a central role (21). Podocytes are terminally differentiated epithelial cells critical to the integrity of the glomerular capillary tuft and form the final barrier to retard passage of macromolecules into the ultrafiltrate (29). Tethered to the outer aspect of the basement membrane, podocytes are particularly vulnerable to distention of the glomerular tuft resulting from elevated intraglomerular pressure. Because podocyte loss is an early event in the pathway to glomerulosclerosis (21), it is logical that the deleterious effects of glomerular capillary hypertension involve podocyte injury. We and others showed that the resultant mechanical strain experienced by podocytes initiates a series of maladaptive responses including impaired proliferative capacity (31), apoptosis (10), and detachment of viable cells (32). Unlike other resident glomerular cells, podocytes have a limited capacity in vivo to replenish injured and lost cells, such that a progressive reduction in podocyte number has been demonstrated in both immune and nonimmune mediated diseases of the glomerulus (23, 28). Thus independent of the nature of the inciting event, glomerular capillary hypertension may serve as a common denominator resulting in further podocyte injury and loss and thus fuel the development of future glomerulosclerosis.

SPARC (secreted protein acidic and rich in cysteine), a member of the family of matricellular proteins, has pleiotropic effects mediating cell-matrix interactions. Through its ability to inhibit mitogenic growth factors such as PDGF, vascular endothelial growth factor (VEGF), and bFGF, SPARC diminishes proliferative capacity in various tissue systems (6). Through direct binding and modification of matrix proteins, SPARC disrupts focal cellular adhesions (5). While SPARC is expressed in highest levels in embryonal tissues, its expression in adult vertebrates is restricted mainly to sites of wound healing and tissue remodeling. However, SPARC is constitutively expressed in podocytes under physiological conditions (1) and is activated in response to immune-mediated injury of the podocyte (13). The elevated levels of SPARC in the podocyte likely accelerate renal injury as evidenced by the milder course of experimental diabetic nephropathy in SPARC-null mice compared with diabetic SPARC wild-type mice (38). Similar results have been reported in patients with diabetic nephropathy where SPARC levels correlate with severity of renal insufficiency and proteinuria (20).

This paper asks whether SPARC may be a putative mediator of podocyte injury in states of intraglomerular capillary hypertension. We make the novel observation that mechanical forces lead to increase production of SPARC by podocytes and delineate the underlying signaling pathways involved.
Harvested by trypsin and collagenase (200 U/ml) digestion at 37°C for analysis. Briefly, cells were washed twice with ice-cold PBS and in control and stretched podocytes at 24 and 48 h by Western blot

Western blot analysis. The protein levels of SPARC were measured in control and stretched podocytes at 24 and 48 h by Western blot analysis. Briefly, cells were washed twice with ice-cold PBS and harvested by trypsin and collagenase (200 U/ml) digestion at 37°C for 10 min. Cells were pelleted by centrifugation (1,400 rpm for 5 min at 4°C), washed twice with ice-cold PBS, and then suspended in lysis buffer containing 1% Triton X-100, 10% glycerol, 20 mM HEPES, 100 mM NaCl and protease inhibitor cocktail (Roche). Following an overnight freeze-thaw cycle, lysates were cleared by centrifugation at 17,000 g for 10 min at 4°C and protein concentration was determined by BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer’s directions. Reducing buffer was added to each protein extract, and samples were boiled for 5 min. Five micrograms of reduced protein sample were then loaded per lane on an 8% SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane (Immobilon-P) by electroblotting at 350 mA for 75 min. After being blocked for 30 min in 5% nonfat dry milk to reduce background, membranes were incubated with primary rabbit polyclonal anti-SPARC antibody 5944 (gift from Dr. H. Sage) overnight at 4°C. This affinity-purified polyclonal antibody has been well characterized previously and produces a discrete band at 43 kDa under reducing conditions (34). Specificity of the anti-SPARC antibody has been confirmed in preincubation studies with SPARC protein (34) as well as immune blotting studies of wildtype and knockout mesangial cells (2). Following three wash cycles with TBST, membranes were incubated with an anti-rabbit alkaline phosphatase-conjugated secondary antibody (1:2,000 dilution, Promega, Madison, WI) for 60 min at room temperature. The chromagen 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma) was used for detection of the resultant bands. Densitometric quantitation was performed using ImagePro-Plus software (Media Cybernetics, Silver Spring, MD) and results were corrected to GAPDH levels, used as a housekeeper, to correct for any potential errors in loading. Recombinant human SPARC (gift from Dr. H. Sage) was used as a positive control.

Tissue levels of SPARC in a disease model of glomerular capillary hypertension. To ensure that the cell culture results also occurred in vivo, the uninephrectomized spontaneously hypertensive rat (SHR) model of glomerular capillary hypertension was utilized. Unilateral nephrectomy was performed on SHR rats at 5 wk of age. Animals were killed 7 and 10 wk following nephrectomy (time points at which elevated intraglomerular pressures have been documented), and the remaining kidney was harvested, fixed in Methacarn, and immunostaining was performed for SPARC. Tissue from age-matched sham-operated rats served as controls. Death of animals for kidney retrieval is done by isofluorane anesthesia followed by cardiac exsanguination and is in accordance with protocols approved by the University of Washington Animal Care Committee.

Briefly, tissue sections were deparaffinized in Histoclear (National Diagnostics, Atlanta, GA), rehydrated with ethanol, and treated with hydrogen peroxide to neutralize endogenous peroxidase. Tissue sections were incubated with primary rabbit polyclonal anti-SPARC
antibody 5944 overnight at 4°C, followed by a biotinylated goat anti-rabbit secondary antibody (1:100 dilution, Promega) for 60 min at room temperature, followed by ABC reagent (Vector Laboratories) for 20 min at room temperature. Color development was achieved by incubating in DAB solution at 37°C for 10 min and counterstaining in methyl green for 2 min. Substitution of the primary antibody with an irrelevant rabbit IgG served as a negative control. SPARC staining was measured using Optimus 6.5 System (Media Cybergenetics) and expressed as the percentage of glomerular area staining positive for SPARC. A minimum of 20 glomeruli were examined per tissue section, and a total of 6 animals were studied in each group at each time point.

To confirm increased intensity of SPARC staining in podocytes, double staining was performed for SPARC and WT-1, a transcription factor expressed exclusively by the podocyte in the adult kidney where it is important in maintaining podocyte differentiation (37). Deparaffinization of tissue sections was carried out as outlined, and staining was performed using a primary polyclonal rabbit anti-WT1 antibody overnight at 4°C. Following application of biotinylated secondary antibody and ABC reagent as above, color development was performed in DAB supplemented with nickel to optimize nuclear staining pattern of WT1. Tissue sections were then incubated in 4% rabbit serum for 60 min at room temperature to saturate available binding sites of the secondary anti-rabbit antibody, followed by goat anti-rabbit F(ab) fragments (Jackson ImmunoResearch Laboratories, West Grove, PA) overnight at 4°C to mask the rabbit anti-WT1 primary antibody. Staining for SPARC was then performed as previously outlined.

**MAPK signaling studies.** Western blot analysis was used to determine which signaling pathways are activated in response to mechanical strain, utilizing antibodies that recognize the phosphorylated forms of p38, ERK, and SAPK/JNK obtained from Cell Signaling Technologies (Beverley, MA). To demonstrate the potential role of these specific pathways in mediating stretch-induced changes in SPARC, studies of growth-restricted podocytes were repeated in the presence of SB-202190 (selective inhibitor of p38 pathway), SP-600125 (selective inhibitor of JNK pathway), PD-98059 (inhibitor of ERK pathway), or DMSO (vehicle). Cells were preincubated in the presence of MAPK inhibitors (all obtained from Sigma) at a final concentration of 10 μM for 60 min before initiating cyclical stretch as previously outlined, and SPARC was measured by Western blot analysis.

**Statistical analysis.** Unless otherwise noted, all experiments were repeated on at least three separate occasions. Western blots were run using protein harvested on each occasion, and densitometric analysis was performed in triplicate on each blot. The results were pooled and presented graphically with error bars representing the standard deviation. Statistical analysis on data obtained was performed using paired t-test or ANOVA with a Bonferroni-Dunn correction (Statview 5.0, Abacus Concepts, Berkeley, CA). A P value <0.05 was considered statistically significant.

**RESULTS**

**Conditionally immortalized mouse podocytes synthesize SPARC.** We first ensured that podocytes grown in culture synthesize SPARC. Conditionally immortalized mouse podocytes were growth restricted for 14 days, and total RNA and protein were isolated from cell lysate. RT-PCR was used to determine SPARC mRNA expression, and the results are shown in Fig. 1A. While substitution of cDNA with water as a negative control produced no bands, a single band at 300 bp (predicted size for SPARC based on primer sequences) was seen with both cDNA from podocytes, as well as the positive control (mouse mesangial cell). As shown in Fig. 1B, using recombinant human protein as a positive control, the presence of SPARC protein was confirmed in whole cell lysate by Western blot analysis. The subcellular localization of SPARC was determined by immunofluorescent staining. As shown in Fig. 1C, while substitution with an irrelevant rabbit IgG produced no staining (left), the anti-SPARC 5944 primary antibody revealed a predominantly granular pattern throughout the cytoplasm with extension into cell processes (right).

**Mechanical strain increases SPARC expression in podocytes in vitro.** Glomerular capillary hypertension characterizes many forms of chronic kidney disease and perpetuates further podocyte injury. We were interested in determining the effects of elevated intraglomerular pressure on the levels of SPARC in podocytes. In an effort to mimic the mechanical strain experienced by podocytes in vivo, growth-restricted podocytes were exposed to cyclical stretch of 10% amplitude. As shown in Fig. 2A, cyclical stretch resulted in a marked increase in SPARC protein levels at 24 and 48 h compared with static controls. Densitometric analysis corrected for the housekeeping gene GAPDH confirmed a 5.9 (P < 0.01)- and 2.0-fold (P < 0.001) increase, respectively, in SPARC levels. Real-time PCR was performed to determine whether the stretch-induced increase in SPARC is under transcriptional control. As shown in Fig. 2B, mechanical strain resulted in a 1.4- and 1.6-fold increase in SPARC mRNA expression at 6 and 24 h, respectively, compared with nonstretched controls (P < 0.001). Taken together, these data suggest that the induction of SPARC in response to mechanical strain is under transcriptional regulation.
Mechanical strain increases SPARC secretion by cultured podocytes. As a member of the matricellular protein family, many of the effects of SPARC are mediated at the interface between cells and matrix constituents. We were therefore interested in determining whether mechanical strain leads to an increase in secretion of SPARC by podocytes in culture. Cell media were collected from stretched and nonstretched podocytes and Western blot analysis was performed for murine SPARC. As shown in Fig. 3, a marked increase in SPARC was detected in the media harvested from stretched cells compared with nonstretched control cells. To correct for any potential discrepancy in cell number under different experimental conditions, densitometric analysis was performed and results were adjusted for total protein content of cell lysate. As shown in Fig. 3, mechanical strain resulted in a 2.6-, 2.2-, and 2.3-fold increase in SPARC secretion at 18, 24, and 48 h, respectively, compared with nonstretched control cells ($P < 0.01$).

Podocyte levels of SPARC are increased in an experimental model of glomerular capillary hypertension. To demonstrate the relevance of our cell culture studies to the in vivo setting, we determined SPARC levels in an experimental model of glomerular capillary hypertension. The uninephrectomized SHR is a well-characterized noninflammatory model of glomerular capillary hypertension, resulting in progressive glomerulosclerosis and proteinuria. Micropuncture studies have reliably demonstrated elevated glomerular pressures beginning ~5 wk following uninephrectomy (11). Accordingly, Methacarn-fixed kidney sections were obtained 7 wk following uninephrectomy in SHR rats. As shown in Fig. 4, low levels of SPARC were detected in the glomerulus of a sham-operated control (A) or a control kidney excised before the development of increased glomerular pressure (B). In contrast, a marked increase in intensity of staining for SPARC is evident in uninephrectomized SHR rat at 7 wk (C, arrows) and is even more pronounced at 10 wk (D, arrows) following uninephrectomy. Specifically, when expressed as percentage of glomerular area (Fig. 4E), an 8.2- and 9.3-fold increase in SPARC staining was observed at 7 and 10 wk in the uninephrectomized SHR rats compared with control animals ($P < 0.001$). Specificity of the antibody was confirmed by the absence of staining following substitution of the primary antibody with an irrelevant rabbit IgG (data not shown).

To confirm increased intensity of SPARC staining in podocytes, double staining was performed for SPARC and WT-1, a transcription factor expressed exclusively by the podocyte in the adult kidney where it is important in maintaining podocyte differentiation. As shown in Fig. 5, nuclear staining for WT-1 (arrowhead, A) colocalizes with SPARC-positive cells in the glomerulus (arrows, B). Taken together, these data are consistent with our in vitro findings and suggest that podocytes are responsive to mechanical strain in states of elevated glomerular capillary pressures, resulting in an increase in SPARC production.
Stretch-induced upregulation of SPARC is p38 dependent. Having demonstrated that mechanical forces increase podocyte levels of SPARC in culture and experimental disease, we determined the signaling pathways involved. Western blot analysis for the phosphorylated (activated) forms of p38, ERK, and JNK was performed on whole cell lysate from stretched and nonstretched control cells. As shown in Fig. 6, when adjusted for total p38 levels as a loading control, densitometric analysis revealed a 2.4 (\(P < 0.02\)), 2.7 (\(P < 0.02\)), and 1.3-fold (\(P = 0.06\)) increase in phospho-p38 at 5, 20, and 40 min, respectively, in stretched cells compared with nonstretched controls. In contrast, mechanical strain did not alter the level of activation of JNK or ERK pathways in podocytes (results not shown).

The p38 MAPK family plays an important role in cellular responses to a variety of external stressors and has been implicated in mediating podocyte injury. To prove a role for p38 in mediating stretch-induced increase in SPARC, growth-restricted podocytes were preincubated for 60 min in media containing SB-202190 (10 \(\mu\)M), a selective inhibitor of the p38 pathway (9), and exposed to pulsatile strain as previously outlined. As shown in Fig. 7, inhibition of the p38 pathway by SB-202190 completely abrogated the increase in SPARC induced by mechanical strain at 48 h. In contrast, incubation with...
an equivalent volume of DMSO (vehicle for the p38 antagonist) had no demonstrable effect on SPARC levels, confirming specificity of action of SB-202190. While we did not observe activation of the ERK or JNK pathways by mechanical stretch, for the sake of completeness, preincubation studies were also performed in the presence of the selective inhibitors SP-600125 and PD-98059, respectively. Preincubation with inhibitors of the JNK pathway or ERK pathway did not have any appreciable effect on preventing the stretch-induced increase in SPARC (data not shown). Taken together, these data suggest that the upregulation of SPARC in response to mechanical strain occurs in a p38-dependent fashion.

**DISCUSSION**

A progressive reduction in podocyte number leads to the development of glomerulosclerosis (22). While the podocyte is a primary target of injury in many forms of glomerular disease such as membranous nephropathy, focal segmental glomerulosclerosis, and diabetic nephropathy, podocytes may be secondarily injured by mechanical forces resulting from glomerular capillary hypertension. Podocytes indeed are mechanosensitive as our group and others previously demonstrated (12). Exposure to excess levels of mechanical strain initiates a series of maladaptive responses. In contrast to mesangial cells that proliferate in response to mechanical forces (18), we showed that cyclical stretch is antiproliferative for podocytes in culture (31). Furthermore, we recently demonstrated that mechanical strain increases SPARC in podocytes through activation of the p38 pathway (31). Therefore, understanding mechanisms by which mechanical forces injure podocytes is of paramount importance.

With the use of a combined in vitro-in vivo approach, the major finding of this study was that mechanical forces characteristic of states of glomerular capillary hypertension lead to increased levels of SPARC in podocytes. Pulsatile strain experienced by podocytes was simulated using vacuum pressure to induce repetitive cell deformation. A regimen of 60 cycles per minute of 10% biaxial elongation was deemed a reasonable approximate of the degree of stretch experienced by podocytes in vivo as recently discussed (10). Exposure of conditionally immortalized mouse podocytes to cyclical stretch markedly increased SPARC levels in whole cell lysate as well as secreted SPARC into the extracellular milieu. Furthermore, mechanical strain increased SPARC mRNA levels, suggesting the stretch-induced increase in SPARC is under transcriptional regulation.

To demonstrate relevance of these findings to the disease state, immunohistochemistry for SPARC was performed in the uninephrectomized SHR. While the SHR strain is considered an animal model of essential hypertension with eventual renal injury restricted to the medullary nephrons, surgical reduction of nephron mass by uninephrectomy results in accelerated renal disease characterized by progressive proteinuria and widespread glomerulosclerosis (11, 39). Furthermore, intraglomerular capillary pressure predictably increases 5 wk following uninephrectomy and is a prerequisite for the subsequent development of glomerular injury (39). The uninephrectomized SHR rat is therefore a useful experimental model of glomerular capillary hypertension. At 7 wk following uninephrectomy, intensity of SPARC staining was increased in a podocyte distribution compared with sham-operated controls. Furthermore, studying different time points in the same animal revealed that before the development of glomerular capillary pressure (i.e., at the time initial nephrectomy was performed) glomerular staining for SPARC was minimal. In contrast, 10 wk following uninephrectomy (a time point at which intraglo-


REFERENCES


MECHANICAL STRAIN INCREASES SPARC IN PODOCYTES

F583

AJP-Renal Physiol • VOL 289 • SEPTEMBER 2005 • www.ajprenal.org
MECHANICAL STRAIN INCREASES SPARC IN PODOCYTES


