Scavengers of reactive oxygen species, paracalcitol, RhoA, and Rac-1 inhibitors and tacrolimus inhibit angiotensin II-induced actions on glomerular permeability

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Submitted 15 March 2013; accepted in final form 5 May 2013

Axelsson J, Rippe A, Sverrisson K, Rippe B. Scavengers of reactive oxygen species, paracalcitol, RhoA, and Rac-1 inhibitors and tacrolimus inhibit angiotensin II-induced actions on glomerular permeability. Am J Physiol Renal Physiol 305: F237–F243, 2013. First published May 8, 2013; doi:10.1152/ajprenal.00154.2013.—Systemic infusions of ANG II rapidly induce large, dynamic increases in the permeability of the glomerular filtration barrier (GFB) in rats. After binding to its receptor(s), ANG II generates reactive oxygen species (ROS) and produces Ca^2+ influx into cells, leading to activation of a plethora of signaling cascades, including, e.g., calcineurin and small GTPases, such as Rac-1 and RhoA. In the present study we sought to interact with some of these cascades to test potential novel antiproteinuric agents. In anesthetized Wistar rats, the left urether was cannulated for urine collection, and blood access was achieved. Rats were infused with ANG II (16 ng·kg^-1·min^-1) alone, or together with the ROS scavengers tempol or dimethyliourea (DMTU) or the D-vitamin analog paracalcitol, the RhoA-kinase inhibitor Y-27632, the Rac-1 inhibitor NSC-23766, or the calcineurin inhibitor tacrolimus. FITC-Ficoll-70/400 (mol.radius 10–80 Å) and 51Cr-EDTA were infused throughout the experiment. Plasma and urine samples were taken during baseline and at 5 and 15 min after the start of the infusions and analyzed by high-performance size-exclusion chromatography for determination of glomerular sieving coefficients (θ) for Ficoll10–80Å. ANG II infusion into rats caused marked increases in glomerular permeability to large Ficoll molecules (Ficoll10–80Å), which were abrogated by the ROS scavengers tempol and partly by DMTU. Paracalcitol, RhoA, and Rac-1 inhibition, and, to some extent tacrolimus, but not procalcyclin, could also inhibit the glomerular permeability actions of ANG II. Our data suggest that cellular ROS generation and active Ca^2+ signaling are involved in ANG II-induced increases in glomerular permeability.

Ficoll; glomerular sieving coefficient; microalbuminuria; capillary permeability; two-pore model

WE HAVE PREVIOUSLY DEMONSTRATED that systemic infusions of ANG II into rats cause rapid and marked increases in the glomerular permeability to macromolecules, largely independently of the concomitant hemodynamic ANG II effects (6). These actions were blocked by the ANG II receptor blocker (ARB) candesartan. Thus ANG II exerts its permeability effects mainly via interaction with the angiotensin type 1 receptor (AT1R), present in both podocytes and endothelial cells of the glomerular filtration barrier (GFB), to cause rearrangements of cellular F-actin, mostly studied in cultured podocytes (23). The interaction of ANG II with AT1R causes Ca^2+ influx into the cells via so-called transient receptor potential canonical (TRPC) channels, TRPC5 and TRPC6, activating a number of Ca^2+ signaling cascades (20). One of the key signaling molecules involved in the ANG II actions is NAD(P)H oxidase (Nox), which promotes reactive oxygen species (ROS) generation, and, in turn, influences many downstream signaling targets, including mitogen activated protein (MAP) kinases, tyrosine kinases, protein phosphatases, transcription factors, and RhoA/Rho kinase, etc. (35). The small GTPase Rac-1 is part of the intricate membrane-bound multienzyme complex that forms activated Nox, generating ROS upon e.g., ANG II stimulation.

Recently, some of the major Ca^2+ signaling pathways that are responsible for podocyte injury were reviewed (20). However, in the review the early steps leading to Nox activation and ROS generation were not discussed. The activation of AT1R was thought to mainly induce TRPC5-driven, and to some extent TRPC6-driven, Ca^2+ influx into the cell, and, next, the Ca^2+ activated phosphate calcineurin or kinases, such as PKA, both of which were assumed to compete for downstream effects on synaptotopin, mainly a target of calcineurin, and on nuclear factor of activated T-cell (NFAT), mainly a target of protein kinases. At the next step, the small GTPases, Rac-1, RhoA, and Cdc42, were suggested to compete for downstream effects on the actin cytoskeleton, inducing either cell contraction and “stiffness” via RhoA activation, or “hypermobility” and possibly foot process (FP) effacement in either cell contraction and “stiffness” via RhoA activation, or “hypermobility” and possibly foot process (FP) effacement in podocytes via Rac-1 activation (20). Based on experimental data (38), it was hypothesized that in states of excess ANG II, TRPC5/Rac-1 overactivity may drive proteinuria, whereas under physiological conditions moderately active TRPC6 channels may be more important (20).

In the present study, we attempted to test some intracellular key mediators of the ANG II effects on glomerular permeability based on the evidence that ANG II initiates ROS generation and intracellular Ca^2+ signaling, affecting cell contractility and GFB dynamics. The strategy was partly guided by clinical results on some novel antiproteinuric agents, other than angiotensin-converting enzyme (ACE) inhibitors or ARB, such as paracalcitol and calcineurin inhibitors. In addition, we wanted to test the effects of ROS inhibitors and also inhibitors of small GTPases, which regulate cytoskeletal actin contractility, on ANG II-induced increases in glomerular permeability. As a “negative control,” systemic prostacyclin (PGI2) administration was also tested in parallel with ANG II infusions.

To assess glomerular permeability in intact rats, we studied the glomerular sieving coefficients (θ), i.e., the primary urine-to-plasma concentration ratios, of FITC-Ficoll 70/400 (Mr = 70,000 and = 400,000, respectively) and of the Stokes-Einstein (SE) radius (a_e) ranging from 10 to 80 Å in rats infused with moderately supraphysiological doses of ANG II
This challenge causes marked increases in the glomerular permeability to Ficoll50 – 80Å. A single dose of 18 mg was given 15 min before the ANG II infusion and was given as a pretreatment infusion only during the first dose (18 mg) was given 15 min before the ANG II infusion and was given as a bolus dose of 10 μg FITC-Ficoll-70, 960 μg FITC-Ficoll-400, 500 μg FITC-Inulin, and 0.3 MBq 51Cr-EDTA was followed by a constant infusion of 10 μl·kg⁻¹·h⁻¹ (FITC-Ficoll-70, 20 μg/ml; FITC-Ficoll-400, 0.48 mg/ml; FITC-inulin, 0.5 mg/ml) for at least 20 min before sieving measurements, after which urine from the left kidney was collected for 5 min, with a midpoint (2.5 min) sample plasma collected.

**Materials and Methods**

**Animals.** Experiments were performed in 42 male Wistar rats (Møllergard, Lille Sтенsved, Denmark) with an average body weight of 263.2 ± 2.2 g. The rats had free access to water and standard chow until the day of the experiment. The animal Ethics Committee at Lund University approved the animal experiments.

**Surgery.** Anesthesia was induced with pentobarbital sodium (60 mg/kg) intraperitoneally (ip), and body temperature was kept at 37°C by a thermostatically controlled heating pad. The tail artery was cannulated (PE-50 cannula) for continuous monitoring of mean arterial pressure (MAP) and heart rate (HR) on a polygraph (model 7B; Grass Instruments, Quincy, MA) and for repeated administration of anesthesia (pentobarbital sodium). The left carotid artery was cannulated (PE-50 cannula) for blood sampling and the left and right jugular veins for infusion purposes (using PE-50 cannulas). Furosemide (Furosemide, 0.375 mg/kg, Recip, Sweden) was administered in the tail artery to increase urine production and facilitate cannulation of the urether. Access to the left urether was obtained through a small (6 – 8 mm) abdominal incision. The urether was dissected free, and a PE-10 cannula (connected to PE-50) was inserted and secured by a ligature.

**Experimental protocol.** All experiments started with an initial resting period of at least 20 min following the cannulation of the left urether. The dose of ANG II (A9525, Sigma-Aldrich, St. Louis, MO) used has been previously tested in the study by Axelsson et al. (6) and was found to enhance the glomerular permeability to Ficoll50 – 80Å and continues throughout the experiment. Another group of rats received the free hydroxyl radical (HO· scavenger, dimethylthioureia (DMTU, Sigma-Aldrich; DMTU-ANG II; n = 6), given as a single dose before the infusion of ANG II. The first dose (18 mg) was given 15 min before the ANG II infusion and started and the second (18 mg) at the onset of the ANG II infusion.

The vitamin D analog, paracalcitol, was given to another group of rats (Zemplar, Abbott Scandinavia, Solna, Sweden) and was administered as a single dose 15 min before the start of the ANG II administration (paracalcit-ANG II; n = 6, a single dose of 0.4 μg/kg).

In a separate group of animals, ANG II was given in combination with the calcineurin inhibitor tacrolimus (Prograf, Astellas Pharma). Administration of tacrolimus started 30 min before the ANG II administration and was given as a pretreatment infusion only during this time period (tacrolimus-ANG II; n = 6, 2 mg/kg for 30 min).

The inhibitors of small GTPases, Rho kinase inhibitor (ROCKi; Y-27632, Mitsubishi Pharma, Osaka, Japan) and Rac-1 inhibitor (Rac-1i; NSC-23766, Calbiochem, San Diego, CA), were given to two separate groups of animals. Both drugs were administered as a bolus and continuous infusion starting 5 min before the start of the ANG II administration (ROCKi-ANG II; n = 6, bolus of 15 ng, followed by an infusion of 8.9 μg·kg⁻¹·min⁻¹, and Rac-1i-ANG II; n = 6, bolus of 12.5 μg, followed by an infusion of 9.3 μg·kg⁻¹·min⁻¹, respectively).

Another group of animals was given prostacyclin (PGI2; Epoprost, Stoln, Glaxo Wellcome Operations, Greenfield, UK). The PGI2 infusion, 2 ng·kg⁻¹·min⁻¹ (PGI-ANG II; n = 6) was started 15 min before the start of the ANG II infusion and was continued throughout the experiment.

**FITC-Ficoll.** A mixture of FITC-Ficoll-70 (10 mg/ml) and FITC-Ficoll-400 (10 mg/ml) (TdB Consultancy, Uppsala, Sweden) in a 1:24 relationship was administered as a bolus dose together with FITC-Inulin (10 mg/ml, TdB Consultancy). The bolus dose (40 μg FITC-Ficoll-70, 960 μg FITC-Ficoll-400, 500 μg FITC-Inulin, and 0.3 MBq 51Cr-EDTA was followed by a constant infusion of 10 μl·kg⁻¹·h⁻¹ (FITC-Ficoll-70, 20 μg/ml; FITC-Ficoll-400, 0.48 mg/ml; FITC-inulin, 0.5 mg/ml) for at least 20 min before sieving measurements, after which urine from the left kidney was collected for 5 min, with a midpoint (2.5 min) sample plasma collected.

**GFR.** GFR was measured in the left kidney during the experiment using 51Cr-EDTA. A priming dose of 51Cr-EDTA (0.3 MBq in 0.2 ml iv, Amersham Biosciences, Buckinghamshire, UK) was administered and followed by a continuous infusion (10 μl·kg⁻¹·h⁻¹) of 51Cr-EDTA (0.3 MBq/ml) throughout the experiment. Urine was collected from the left urether repeatedly during this period, and blood samples, using microcapillaries, were taken for calculating GFR, approximately every 5–10 min. Radioactivity in blood and urine was measured in a gamma counter (Wizard 1480, LKP, Wallac, Turku, Finland). Hematocrit was assessed throughout the experiments so as to convert blood radioactivity into plasma radioactivity. During theFITC-Ficoll sieving period, GFR was also assessed from the urine clearance of FITC-inulin (results not shown).

**High-performance size-exclusion chromatography.** A HPLC system (Waters, Milford, MA) was used to determine size and concentration of the Ficoll samples. Size exclusion was achieved using an Ultrahydrogel-500 column (Waters) connected to a guard column (Waters). The mobile phase was driven by a pump (Waters 1525), and fluorescence was detected with a fluorescence detector (Waters 2475) with an excitation wavelength set at 492 nm and an emission wavelength at 518 nm. The samples were loaded to the system with an autosampler (Waters 717 plus), and the system was controlled by Breeze Software 3.3 (Waters). The column was calibrated with Ficoll, and protein standards were described at some length in a previous paper (2).

**Calculations.** The urinary excretion of 51Cr-EDTA and/or FITC-inulin per min (Uᵢ × Vᵢ) divided by the concentration of tracer in plasma (Pᵢ) was used to calculate GFR where Uᵢ represents the tracer concentration in urine and Vᵢ the flow of urine per minute. Ficoll 0 were obtained by analyzing high-performance size-exclusion chromatography (HPSEC) curves from the plasma (Cpᵢ) and urine sample for each experiment. The urine concentration-vs.-Stokes-Einstein radius (aᵢ) curve was divided by the inulin concentration to obtain the plasma/inulin concentration (Cuᵢ). Calculation of 0 for each aᵢ was done by dividing Cuᵢ by Cpᵢ. A two-pore model (28, 33, 34) was used to analyze the data for Ficoll (molecular radius 10–80 Å). A nonlinear least-squares regression analysis was used to obtain the best curve fit, using scaling multipliers, as described at some length previously (34).

**Statistical analysis.** Values are presented as means ± SE. Differences among groups were tested using nonparametric analysis of variance with the Kruskal-Wallis test and post hoc tested using the Mann-Whitney U-test. Bonferroni corrections for multiple comparisons were made when applicable. Significance levels were set at *P < 0.05, **P < 0.01, and ***P < 0.001. All statistical calculations were performed with SPSS 18 (SPSS, Chicago, IL).
were made using IBM SPSS Statistics 20.0 for Windows (SPSS, Chicago, IL).

RESULTS

Effects of ROS inhibition on ANG II-induced increases in glomerular permeability. Figure 1 shows the glomerular θ for Ficoll70Å plotted vs. time for ANG II alone (hatched line) or in the ANG II groups treated with either tempol (solid line) or pretreated DMTU (dotted line), respectively. Tempol totally abolished the effects of ANG II at 5 and 15 min, while DMTU reduced its permeability effects at 15 min, but not at 5 min. At 5 min, θ for Ficoll70Å thus increased from $2.99 \times 10^{-5}$ to $1.15 \times 10^{-5}$ (baseline) to $1.53 \times 10^{-4} \pm 5.77 \times 10^{-5}$ ($P < 0.05$) in ANG II. In tempol-ANG II, θ for Ficoll70Å remained at $2.84 \times 10^{-5} \pm 1.51 \times 10^{-5}$ (not significant), and in DMTU-ANG II it increased to $1.07 \times 10^{-4} \pm 2.26 \times 10^{-5}$ ($P < 0.05$) at 5 min, but declined to near baseline at 15 min ($P < 0.05$ vs. ANG II at 15 min). GFR (Fig. 2), MAP, and HR (data not shown) were stable in the experimental groups throughout the experiments.

Effects of paracalcitol. The effect of paracalcitol on the ANG II-induced increases in glomerular permeability is shown in Fig. 3. Paracalcitol more or less totally abrogated the ANG II effects on glomerular permeability for Ficoll70Å. Thus θ for Ficoll70Å remained at baseline level at both 5 and 15 min after the start of the ANG II infusion, i.e., at $3.14 \times 10^{-5} \pm 9.44 \times 10^{-6}$ and $2.58 \times 10^{-5} \pm 8.20 \times 10^{-6}$, at 5 and 15 min, respectively. Paracalcitol did not affect either GFR (Fig. 2), MAP, or HR (data not shown) throughout the experiment.

Effects of calcineurin inhibition. The calcineurin inhibitor tacrolimus only moderately affected ANG II-induced increases in glomerular permeability for Ficoll70Å at 5 min after the start of the ANG II infusion (Fig. 4). However, moderate inhibition of the ANG II response was seen at 15 min. Between 5 and 15 min, θ for Ficoll70Å was thus reduced from $7.29 \times 10^{-5} \pm 3.34 \times 10^{-5}$ to $4.48 \times 10^{-5} \pm 1.56 \times 10^{-5}$, the reduction being of borderline significance ($P = 0.063$), compared with ANG II at 15 min, where θ for Ficoll70Å was $1.14 \times 10^{-4} \pm 4.39 \times 10^{-5}$. No changes in MAP or HR (data not shown) or GFR (Fig. 2) were observed.

Effects of Rho-kinase and Rac-1 inhibition. The RhoA kinase inhibitor ROCKi and the Rac-1 inhibitor Rac-1i effectively inhibited the increase in θ for Ficoll70Å by ANG II at 5 and 15 min, as shown in Fig. 5. Ficoll70Å θ thus remained at baseline, i.e., at $1.91 \times 10^{-5} \pm 6.95 \times 10^{-6}$ ($P < 0.01$ compared with ANG II) and $2.61 \times 10^{-5} \pm 8.38 \times 10^{-6}$ ($P < 0.01$ compared with ANG II) in ROCKi-ANG II and Rac-1i-ANG II, respectively, at 5 min. Simultaneous infusion of
ROCKi or Rac-li with ANG II caused no significant changes in MAP, HR (results not shown), or GFR (Fig. 2).

Effects of PGI2 infusion. PGI2 had no effect on the ANG II-induced increase in glomerular permeability for Ficoll50–80Å at 5 and 15 min (Fig. 6), since the ANG II-induced glomerular hyperpermeability prevailed at both these time points. The simultaneous infusion of PGI2 and ANG II did not alter MAP or HR (data not shown). Furthermore, GFR (Fig. 2) was stable throughout the experiments.

Two-pore parameters. The best curve fits of $\theta$ vs. $r_o$ for Ficoll according to the two-pore model were obtained with respect to the five major parameters of this model: small-pore radius ($r_s$), large-pore radius ($r_l$), the fractional hydraulic conductance accounted for by the large pores ($\alpha_l$), the fractional fluid flow through the large pores ($J_{vl}/GFR$), and the unrestricted pore area over unit diffusion path length ($A_o/\Delta X$).

Statistical comparison of test values of these parameters was performed against their “baseline” period values, each animal serving as its own control. There were, however, no detectable changes in $r_s$, $r_l$, or $A_o/\Delta X$ in any group given inhibitor drugs, except for changes in $\alpha_l$ (and $J_{vl}/GFR$) in the DMTU-ANG II group at 5 min. Thus, in DMTU-ANG II, $\alpha_l$ increased from $3.08 \times 10^{-5} \pm 0.44 \times 10^{-5}$ to $7.51 \times 10^{-5} \pm 2.47 \times 10^{-5}$ ($P < 0.01$), indicating that DMTU had not prevented the increased number of large pores induced by ANG II at 5 min.

In the tacrolimus-ANG II group, in which glomerular permeability changes were barely prevented at 5 min, any significant changes in $J_{vl}/GFR$ or $\alpha_l$ were, however, not seen. For the PGI2-ANG II group, a statistically significant increase in $r_l$ was observed at both 5 and 15 min, $r_l$ increasing from $122 \pm 9.17$ to $161 \pm 5.48$ ($P < 0.01$) and $167 \pm 11.4 \AA$ ($P < 0.05$), at 5 and 15 min, respectively.

DISCUSSION

Systemic infusions of ANG II into rats cause rapid, dynamic changes in the glomerular permeability to macromolecules (6). In the present study, we sought to interact with some of the mechanisms involved in these acute increases in glomerular permeability. Nearly two decades ago, it was observed that ANG II has the ability to activate Nox and to generate ROS in vascular smooth muscle cells (18, 21) and that ROS can modify the activity of several intracellular signaling pathways, including tyrosine kinases/phosphatases, MAP kinases, and transcription factors, to induce various cellular events, such as cell contraction, migration, inflammation, growth, and apoptosis (35). In a recent review, AT1R-induced Ca$^{2+}$ influx to the cell (denoted “level 2”), activation of TRPC5 and TRPC6 channels was assumed to result in Ca$^{2+}$ influx to the cell (denoted “level 4”). On the next level (denoted “level 3”), Ca$^{2+}$-activated phosphatases, such as calcineurin, or kinases, such as PAK, were suggested to affect downstream targets, such as synaptopodin and NFAT ("level 4"). Even further downstream ("level 5"), the small GTPases,
Rac-1, RhoA, and Cdc42, were predicted to compete for downstream effects on the actin cytoskeleton and the mineralocorticoid receptor (MR) as well as promoting the recruitment and insertion of TRPC5 into the cell membrane via Rac-1 in a feed-forward loop (“level 6”). Furthermore, increased levels of ANG II have been shown to increase the expression of membrane TRPC6 channels (“level 6”) via calcineurin/NFAT activation (on “level 4”) (14, 32). It was hypothesized, however, that in states of excess ANG II, TRPC5/Rac-1 overactivity may drive proteinuria, but that under physiological conditions, active TRPC6 channels might be more important for podocyte force generation and resilience via RhoA-dependent effects on the actin cytoskeleton (38). This tentative model was presented as a framework for understanding the actions of some novel antiproteinuric agents which have been studied in vitro in cultured podocytes or found effective in clinical trials. Such agents include calcineurin inhibitors, paracalcitol, and Rac-1, or RhoA kinase inhibitors. In the present study, we attempted to test these agents, as well as ROS scavengers, for their tentative ability to reverse acute episodes of glomerular hyperpermeability induced by ANG II. We also tested prostacyclin (PGI2), which is predicted to have no antipermeability effects in states of excess ANG II.

A primary step in the action of ANG II, after its binding to its receptor(s), is the formation of ROS. In the present study, tempol, primarily a superoxide radical (O2−) scavenger, was more efficient in preventing ANG II-induced glomerular hyperpermeability than was DMTU, primarily a hydroxyl radical (HO•) scavenger. ANG II stimulates the production of O2− by a variety of isoforms of Nox, of which Nox2 and P22phox (where “phox” stands for phagocyte oxidase) have been found to be present in podocytes (19). The very short-lived O2− radical is normally rapidly converted to the much less reactive metabolite H2O2 by endogenous dismutases, which, however, may be overwhelmed in states of e.g., excess ANG II. H2O2 is tightly regulated by intracellular and extracellular enzymes, including catalase, glutathione peroxidase, thioredoxin, and other peroxiredoxins, which detoxify H2O2 by converting it to water and O2 (and to other metabolites). Alternatively, H2O2 can be metabolized to secondary metabolites, such as the highly reactive HO− radical. The relative efficacy of tempol in preventing ANG II-induced permeability increases in the present study underscores the primary role of O2− generation in response to ANG II, whereas the lesser effect of DMTU indicates a more subordinate role of (downstream) HO− generation in these ANG II-induced actions. It should, however, be pointed out that this conclusion is dependent upon the specificity of the ROS scavengers tested, particularly that of DMTU. In an in vitro system where a hypoxanthine-xanthine oxidase (HX/XO) system was used to generate O2− and a H2O2-FeSO4 system (0.1 mmol/l FeSO4) was used to generate H2O2 and HO−, both SOD (cf. tempol) and DMTU showed high specificities. Thus in the HX/XO system DMTU showed only a very slight antioxidative effect where SOD was highly efficient, whereas in the H2O2-FeSO4 system DMTU, but not SOD, was highly potent (25).

Nearly a decade ago, it was demonstrated that TRPC6 gain-of-function mutations are associated with a hereditary form of FSGS (focal segmental glomerulosclerosis) (43). TRPC6 also seems to play a role in acquired forms of proteinuric diseases (31). Vitamin D and vitamin D analogs, such as paracalcitol, have antiproteinuric effects, as amply demonstrated, e.g., in the so-called VITAL study (13). Recent studies indicate that vitamin D can reduce proteinuria by affecting TRPC6 expression in podocytes. In vitro cell injury induced by adriamycin in cultured podocytes thus caused an increased TRPC6 expression, while it was reduced with vitamin D treatment (36). Thus it seems that vitamin D, or vitamin D analogs, can downregulate enhanced TRPC6 expression. In the present study, paracalcitol completely abrogated the acute permeability-enhancing effects of ANG II, and it is speculated that this action may partly involve interactions with TRPC6 channels, conceivably via a reduction in the recycling (or expression) of TRPC6 (14, 32). These data seem to argue against a prominent role of TRPC5 activation/recruitment in the acute permeability effects induced by ANG II. Alternatively, the antiproteinuric effect of paracalcitol may be more complex than just affecting routes of Ca2+ influx into the cell. Indeed, the long-term beneficial effects of paracalcitol on proteinuria have usually been ascribed to suppression of renin transcription or to antifibrotic effects (16). Thus the exact nature of the actions of paracalcitol on acute ANG II hyperpermeability actions warrants further investigation.

The calcineurin inhibitors tacrolimus and cyclosporine are well-known immunosuppressive agents, used in e.g., renal transplantation. They are classically thought to interact with NFAT signaling in T cells, which, among other things, results in reduced release of cytokines, mainly IL-2, being critical in the recognition of “non-self” (22). It has been known for a long time that calcineurin inhibitors can also induce remission in proteinuria, e.g., in minimal change disease and FSGS, in which T cell activation is supposed to occur (30). However, calcineurin inhibitors are effective also in a number of other albuminuric diseases, such as Alport’s syndrome (10) and membranous nephropathy (1, 9, 39). It was thus postulated that podocytes might be a direct target of calcineurin inhibitors, independently of NFAT or IL-2 inhibition (15). Thus calcineurin inhibitors (e.g., cyclosporine) can block the calcineurin-mediated dephosphorylation of synaptotagmin, protecting synaptotagmin from cathepsin L-mediated degradation, thereby preserving a “stable” filtration barrier. Indeed, in the previously mentioned study proposed by Greka and Mundel (20), calcineurin-mediated dephosphorylation of synaptotagmin was depicted as a key step in producing proteinuria. In the present experiments, tacrolimus was not able to prevent the immediate increase in ANG II-induced glomerular hyperpermeability, but inhibition tended to occur after ~15 min. The effect of calcineurin inhibition on the acute ANG II-induced glomerular hyperpermeability was thus found to be time dependent and less pronounced than that observed with tempol, paracalcitol, or inhibitors of small GTPases.

The Rho family of small GTPases (RhoA, Rac-1, and Cdc42) controls signal transduction pathways and influences many types of cell activity, particularly actin dynamics. In capillary endothelium, Rac-1, and, to some extent, Cdc42, are the main GTPases required for barrier maintenance and stabilization of adherens junctions, whereas RhoA negatively influences barrier properties (37, 42). RhoA kinase mediates inactivation of myosin light chain (MLC) phosphatase, leading to increased MLC phosphorylation and actin-myosin interaction to produce endothelial cell contraction and interendothelial gap formation and capillary hyperpermeability (37). By contrast, in
podocytes the classic view has until now been that Rac-1 and Cdc42 promote cell mobility, and thereby “hypermobile” podocytes that can produce FP effacement, whereas RhoA promotes the formation of contractile stress fibers in the cell body, thought to be “cell stabilizing” and “barrier protective” (38). However, excessive RhoA activity has indeed been linked to proteinuria (41, 45) and in (experimental) models of glomerulonephritis and diabetes mellitus RhoA inhibition has been found to ameliorate albuminuria (24, 40). From the present data it is highly likely that RhoA-mediated actin reorganization might be involved in acute ANG II-induced glomerular hyperpermeability. Indeed, RhoA kinase inhibition completely abrogated the acute ANG II-induced permeability increases. It has furthermore been shown that Rac-1 antagonistically regulates RhoA and that Rac-1 and Cdc42 may be actively involved in FP effacement (20, 38). In this context, it should be pointed that FP effacement may just be a secondary phenomenon associated with proteinuria and that it may not be causative in the process of creating glomerular hyperpermeability (26). Indeed, the initial permeability increase induced by ANG II studied here is likely to depend on the active contractile machinery of endothelial cells and podocytes primarily regulated by RhoA. By contrast, chronic glomerular hyperpermeability may lead to FP effacement, which would be governed by Rac-1 activation (26). It should also be pointed out that Rac-1 is a cofactor in the active Ncox enzyme complex and that Rac-1 inhibition may prevent ANG II-induced proteinuria by inhibiting Nox and ROS generation. At any rate, Rac-1 inhibition was as efficient as RhoA inhibition in the present study in preventing ANG II-induced increases in glomerular permeability. The specificities of both the RhoA kinase inhibitor and the Rac-1 inhibitor used in the present study have been shown to be quite high (12, 17).

There is good evidence that low-dose prostacyclin can restore an increased vascular protein permeability after trauma (in skeletal muscle) (7) and that low-dose prostacyclin can improve cerebral cortical perfusion during experimental brain injury in rats (8). By contrast, arachidonic acid metabolites (eicosanoids), such as cyclooxygenase products, are mostly known to be detrimental to the glomerular protein permeability barrier (29). Although in a few studies prostacyclin, and prostacyclin analogs, have been reported to ameliorate experimental glomerulonephritis (27, 44), PGI$_2$ was not a priori predicted to be protective with respect to acute ANG II-induced increases in glomerular permeability. Indeed, PGI$_2$ failed to cause any inhibition of the ANG II-induced permeability actions, and it may therefore be regarded as a “negative control” in this context.

The exact nature of the dynamic actions of ANG II and by other glomerular permeability enhancers that we have tested earlier (3–5) on the GFB is not known. The current view is that all three major layers of the GFB, i.e., the endothelium with its glyocalyx, the GBM, and the podocyte FPs with their slit diaphragms, actively contribute to glomerular barrier properties to prevent proteinuria. The rapid permeability response to ANG II showed a dynamic pattern similar to that seen for ANG II on postcapillary venular endothelium (11). Furthermore, the full abrogation of RhoA inhibition of ANG II-induced glomerular hyperpermeability mimics that which has been observed during hyperglycemic glomerular hyperpermeability (4). This may suggest that ANG II may actually exert its major effect on the glomerular endothelium rather than on the podocytes. Furthermore, we have previously shown that the major sieving barrier of the GFB would not be at the level of the podocyte slit diaphragm (28, 34). However, the podocyte barrier may still be crucial, because changes in the podocyte actin cytoskeleton may sublimely alter the shape of the podocytes and the tension that they exert on the GBM, thereby affecting more uphill layers of the GFB. In addition, integrins linking the podocyte FPs to the GBM may be acutely involved in the rapid increases in glomerular permeability seen with ANG II and with other agents that enhance glomerular permeability.

In summary, systemic infusions of ANG II into rats caused marked increases in glomerular permeability to large Ficoll molecules, changes which were abrogated by ROS scavengers, paracalcitols, RhoA and Rac-1 inhibition, and, to some extent, by a calcineurin inhibitor. This is to our knowledge the first time these direct antagonistic effects on ANG II-induced acute glomerular hyperpermeability are demonstrated in vivo. By contrast, PGI$_2$ was not effective in inhibiting glomerular hyperpermeability induced by ANG II. Our data thus suggest that ROS generation and active Ca$^{2+}$ signaling, engaging calcineurin and small GTPases, are involved in the acute dynamic changes in glomerular permeability induced by ANG II. These findings may be important in the search for novel strategies and drugs for ameliorating proteinuria, and hence, disease progression, in various glomerulopathies.

ACKNOWLEDGMENTS

Kerstin Wihlborg is gratefully acknowledged for skillful typing of the manuscript. We are grateful to Prof. Per-Olof Grünke, Lund University, for supplying us with the Rho-kinase inhibitor Y-27632 and with PGI$_2$.

GRANTS

This study was supported by grants from the Swedish Medical Research Council (Grant 08285), the Swedish Heart and Lung Foundation, and the Medical Faculty at Lund University (ALF-grant).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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