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Eluate Derived by Extracorporal Antibody-Based Immunoadsorption Elevates the Cytosolic Ca^{2+} Concentration in Podocytes via B_2 Kinin Receptors

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Key Words
Bradykinin · Cytosolic calcium · Immunoadsorption · Podocytes · Recurrent focal segmental glomerulosclerosis · Renal transplantation

Abstract
Background/Aim: Patients with idiopathic focal segmental glomerulosclerosis (FSGS) often develop a recurrence of the disease after kidney transplantation. In a number of FSGS patients, plasmapheresis and immunoadsorption procedures have been shown to transiently reduce proteinuria and are thought to do this by eliminating a circulating factor. Direct cellular effects of eluates from immunoadsorption procedures on podocytes, the primary target of injury in FSGS, have not yet been reported. ***Methods:*** Eluates were derived from antibody-based immunoadsorption of a patient suffering from primary FSGS, a patient with systemic lupus erythematosus, and a healthy volunteer. The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) of differentiated podocytes was measured by single-cell fura-2 microfluorescence measurements. Free and total immunoreactive kinin levels were measured by radioimmunoassay. ***Results:*** FSGS eluates increased the $[Ca^{2+}]_i$ levels concentration dependently (EC_{50} 0.14 mg/ml; n = 3–19). 1 mg/ml eluate increased the $[Ca^{2+}]_i$ values reversibly from 82 ± 12 to 1,462 ± 370 nmol/l, and then they returned back to 100 ± 16 nmol/l (n = 19). The eluate-induced increase of $[Ca^{2+}]_i$ consisted of an initial Ca^{2+} peak followed by a Ca^{2+} plateau which depended on the extracellular Ca^{2+} concentration. The eluate-induced increase of $[Ca^{2+}]_i$ was inhibited by the specific B_2 kinin receptor antagonist Hoe 140 in a concentration-dependent manner (IC_{50} 2.47 nmol/l). In addition, prior repetitive application of bradykinin desensitized the effect of eluate on $[Ca^{2+}]_i$. A colonic epithelial cell line not reacting to bradykinin did not respond to eluate either (n = 6). Similar to FSGS eluates, the eluate preparations of both the systemic lupus patient and the healthy volunteer led to a biphasic, concentration-dependent $[Ca^{2+}]_i$ increase in podocytes which again was inhibited by Hoe 140. Free kinins were detected in all eluate preparations. ***Conclusion:*** The procedure of antibody-based immunoadsorption leads to kinin in the eluate which elevates the $[Ca^{2+}]_i$ level of podocytes via B_2 kinin receptors.

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Introduction

At present, the pathogenesis of idiopathic focal segmental glomerulosclerosis (FSGS) is undefined. A number of observations led to the assumption that a circulating factor or circulating factors might cause the disease: (1) up to 40% of the FSGS patients develop a recurrence of the disease early after kidney transplantation [1]; (2) infants of mothers with therapy-resistant FSGS show a transient nephrotic syndrome directly after delivery [2]; (3) injection of plasma of FSGS patients induces proteinuria in rats [3]; and (4) plasmapheresis of FSGS patients ameliorates proteinuria [4]. Dantal et al. [5] demonstrated that protein adsorption by protein A Sepharose columns reduced the urinary protein excretion of patients with FSGS and, in addition, that the respective eluates caused albuminuria in rats. Later on, Savin et al. [6] showed that FSGS patient serum, but not serum from patients suffering from other glomerular diseases, led to an increased albumin permeability of glomeruli in vitro. Recently, the eluate activity putatively responsible for this effect was further characterized and called 'the FSGS factor' [7]. With regard to plasma factors putatively involved in the pathogenesis of FSGS, recent reports focused on serum components capable of inhibiting the plasma permeability [8, 9]. Here, it was suggested that an imbalance between permeability factors and their inhibitors due either to increased production and/or urinary loss may constitute a central event in FSGS [10].

Despite all these findings, the exact nature of the circulating factor thought to induce FSGS has not yet been defined. Moreover, Esnault et al. [11] showed that immunoadsorption (IA) also efficiently reduced proteinuria caused by a number of other diseases: membranous nephropathy, diabetes mellitus, IgA nephropathy, and amyloidosis. Based on their data, these authors suggested that IA removes a nonspecific circulating hemodynamics-altering or permeability-increasing factor.

The target of the circulating factor postulated to cause FSGS is not known at present. Recent studies focused on the role of podocyte damage in the development of glomerular sclerosis in general [12]. Furthermore, the podocyte was defined as primary target of injury in FSGS [13]. Owing to the lack of a reliable model to study the podocyte physiology in vitro, up to now no data are available addressing possible mechanisms of podocyte damage in FSGS. Recently, mouse podocyte cell clones have been established, expressing specific proteins of differentiated, nonproliferating podocytes [14], such as the Wilms' tumor gene product WT-1 located in the nucleus [15] or the protein synaptopodin located in the foot processes [16]. Podocytes in culture thus provide a promising tool to investigate a potential direct cellular alteration by the putative FSGS-causing factor(s).

In the present study, we investigated the effect of eluates from antibody-based IA procedures on the free cytosolic Ca^{2+} concentration of differentiated podocytes.

Subjects and Methods

Patient with Primary FSGS

The male patient suffered from nephrotic syndrome due to biopsy-proven FSGS which led to end-stage renal insufficiency. Shortly after a cadaveric kidney transplantation, the patient developed nephrotic syndrome again. A kidney transplant biopsy again showed FSGS which subsequently led to graft loss. After a second kidney transplantation, consisting of pediatric en bloc kidneys localized intra-abdominally, the patient first demonstrated a good transplant function. However, shortly after, nephrotic syndrome occurred again accompanied by a deterioration of the excretory kidney function which was most likely due to recurrent FSGS also in the second transplant. As all medical treatment with diverse immunosuppressive agents (steroids, chlorambucil, cyclophosphamide, ciclosporin A) did not improve the clinical course, after written consent an antibody-based IA therapy was begun using antihuman IgG cartridges. During the course of this treatment, the renal function improved, and proteinuria declined by more than 50%. During IA, medication did not include an angiotensin-converting enzyme (ACE) inhibitor. Eluate derived by IA from this patient increased the albumin permeability of the glomeruli in the test system described by Savin et al. [17].

Patient with Systemic Lupus Erythematosus (SLE)

The female patient suffered from SLE associated with lupus nephritis, leading to end-stage renal insufficiency. Six years after renal transplantation, SLE flared up (antinuclear antibody titer 1:1,600) despite immunosuppression. Upon deterioration of the renal function, transplant biopsy showed recurrent lupus nephritis in combination with acute rejection. As all subsequent medical treatments with diverse immunosuppressives did not improve her clinical course, after written consent antibody-based IA therapy was begun, using antihuman IgG cartridges.

Healthy Volunteer

After written consent, the healthy male individual from the medical staff of the Department of Nephrology underwent a single antibody-based IA treatment, using antihuman IgG cartridges. Three apheresis cycles were performed, as described below.

Extracorporeal Antibody-Based IA

Blood was drawn from a Cimino shunt or large (ante)cubital veins via a dialysis needle (15 gauges; Bionic, Friedrichsdorf, Germany) at a flow rate of 100–120 ml/min. Heparin was added for anticoagulation (1,000 U/h). Plasma separation was performed with a miro-sorb system (Baxter, Unterschleissheim, Germany) and a hollow-fiber plasmapheresis filter (polypropylene; Hemaplex BT 900, 0.4 m^2; Dideco, Mirandola, Italy). Removal of immunoglobulins was accomplished with two sterile and pyrogen-free columns, each containing

polyclonal sheep antibodies against human immunoglobulin (IgG, IgA, and IgM) heavy and light chains conjugated to cyanogen bromide activated Sepharose CL-4B beads (Ig-Therasorb, Baxter). Each column had an immunoglobulin-binding capacity of approximately 4.0 g. In each apheresis cycle lasting 15 min, 250 ml of plasma was loaded on one column (plasma flow 50–60 ml/min), while the other column was regenerated, followed by the same procedure using the second column (total plasma volume/cycle: 500 ml). 10–14 cycles were performed within one IA session lasting 2.5–3.5 h. Therefore, a total of 5–7 liters plasma passed the columns in each session. The columns were regenerated by protein elution with glycine buffer at pH 2.8, followed by washing cycles with phosphate-buffered saline and 9 g/l isotonic sodium chloride solution, pH 7.4. The miro-sorb system allowed for automated control of plasma fluid and regeneration of solutions in the IA columns. After passing the IA columns, patient plasma and separated blood cells were reinfused into the patient either through the shunt or a contralateral (ante)cubital vein. Each pair of reusable columns was assigned to 1 single patient. Columns were kept filled with storage buffer (phosphate-buffered saline, 0.01% sodium azide, pH 7.2) at 10°C between the IA sessions.

Preparation of Eluates for in vitro Experiments

Each eluate portion was filled in a modified cellulose tubing and dialyzed for 48 h at 8°C against bidistilled water. Thereafter, eluates were lyophilized and stored at –40°C until use. For use, the lyophilisates were resuspended in a Ringer-like solution.

Generation and Culturing of Podocyte Cell Lines

The culturing of podocyte cell lines has been described in detail previously [18]. Immortalized mouse podocytes were derived from mice that have a thermosensitive variant of the SV40-T antigen inserted into the mouse genome. These mouse podocytes proliferate at 33°C and in the presence of interferon gamma (SV40-T antigen active). At 37°C and after removal of interferon gamma, the cells transform into the quiescent, differentiated phenotype [18]. Within the current study, the cells stained positive for the podocyte markers WT-1 [15], synaptopodin [19], nephrin [20], and p57 [21]. In addition, mRNA expression of the podocyte marker CD2AP [22] was detected by RT-PCR. For experiments, cells between passages 15 and 25 were seeded at 37°C into six-well plates and cultured in standard RPMI media containing 1% fetal calf serum (Boehringer Mannheim, Germany) and 100 U/ml penicillin and 100 mg/ml streptomycin (both Gibco, Eggenstein, Germany) for at least 14 days, until the cells were differentiated, showing an arborized morphology.

Culturing of Human Colonic Epithelial Cells

The human colonic epithelial cell line HT_{29} was kindly provided by Prof. R. Greger, Department of Physiology, University of Freiburg, Germany. Its culturing was performed as recently described [23].

Measurements of the Free Intracellular Calcium Concentration ($[Ca^{2+}]_i$)

Measurements of $[Ca^{2+}]_i$ with the Ca^{2+}-sensitive fluorescent dye fura-2/AM (5 μM; Molecular Probes, Portland, Oreg., USA) were performed in single podocytes with an inverted fluorescence microscope setup in a modified version from that recently described in detail [24]. In brief, the light from a 75-watt xenon lamp (Osram, Köln, Germany) was directed through a tempax filter to avoid thermal damage of the three excitation filters (Lys and Optiks, Lyngby, Denmark) mounted on a motor-driven filter wheel (10 cycles/s). The excitation filters were 10-nm bandpass filters with transmission maxima at 340, 360, and 380 nm. A dichroic mirror (FT425; Zeiss, Oberkochen, Germany) and a bandpass filter (500–530 nm, Lys and Optiks) were used in the emission light pass. The fluorescence field of a phototube (Hamamatsu H3460, Herrsching, Germany) could be chosen by means of an adjustable rectangular diaphragm (50–150 × 30 μm). After amplification, the photocurrent of the photocounter was digitized with a 12-bit A/D converter (MPC 64, Zeiss) and recorded continuously by means of a personal computer. The fluorescence values for the three excitation wavelengths for each turn of the wheel were computed. In 20 experiments, the calibration of the fura-2 fluorescence signal could be successfully performed at the end of the protocol, using the Ca^{2+} ionophore ionomycin (5 μmol/l) and low- and high-Ca^{2+} buffers, as described previously in detail [24]. $[Ca^{2+}]_i$ was calculated according to the equation described by Grynkiewicz et al. [25]. A K_D for the fura-2-Ca^{2+} complex of 224 nmol/l (37°C) was assumed. The given concentrations for the Ca^{2+} peak refer to the highest magnitude of the fluorescence ratio. Data are mainly given as fluorescence ratio 340/380 nm.

Determination of Free and Total Immunoreactive Kinin

Lyophilized eluate was dissolved in deionized water; insoluble material was removed by centrifugation for 5 min at 10,000 *g*. Concentrations of free and total immunoreactive kinins in these solutions were determined analogously to a method described previously [26]. In brief: For determination of free kinin, 0.4 ml of the solution was mixed with 1.6 ml absolute ethanol, the precipitate was removed by centrifugation, and the supernatant was evaporated to dryness. For determination of total kinin, an aliquot of the solution was incubated with trypsin before ethanolic extraction. The dry residue was dissolved in radioimmunoassay buffer, and the kinin concentration was determined by a radioimmunoassay using antibodies specific for kinins [27]. In a second set of experiments, determination of the kinin concentrations was performed after lyophilized eluate was dissolved in a Ringer-like solution supplemented with 2 mmol/l EDTA (final eluate concentration 1 mg/ml). In a third set of experiments, podocytes were incubated with lyophilized eluate dissolved in a Ringer-like solution (0.2 ml, final eluate concentration 1 mg/ml) for 4 or 40 min. In a fourth set of experiments, podocytes were incubated with lyophilized eluate dissolved in a Ringer-like solution supplemented with 2 mmol/l EDTA (final eluate concentration 1 mg/ml) for the same time periods. In each series, supernatants then were removed and mixed with 1.6 ml absolute ethanol. The preparation of the different samples for kinin measurements was performed as described above. EDTA was added in the second and fourth set of experiments in order to inhibit kininases which might be present in the eluate, on the outer cell membrane, or be released from podocytes.

Chemicals

Chemicals were of the highest grade of purity available and were obtained from Sigma unless otherwise indicated.

Statistics

The data are presented as mean values ± SEM (n), where *n* refers to the number of experiments. A paired t test was used to compare mean values within one experimental series. $p \leq 0.05$ was accepted to indicate statistical significance. Concentration-response curves were calculated by nonlinear regression analysis using the Hill formula with four parameters.

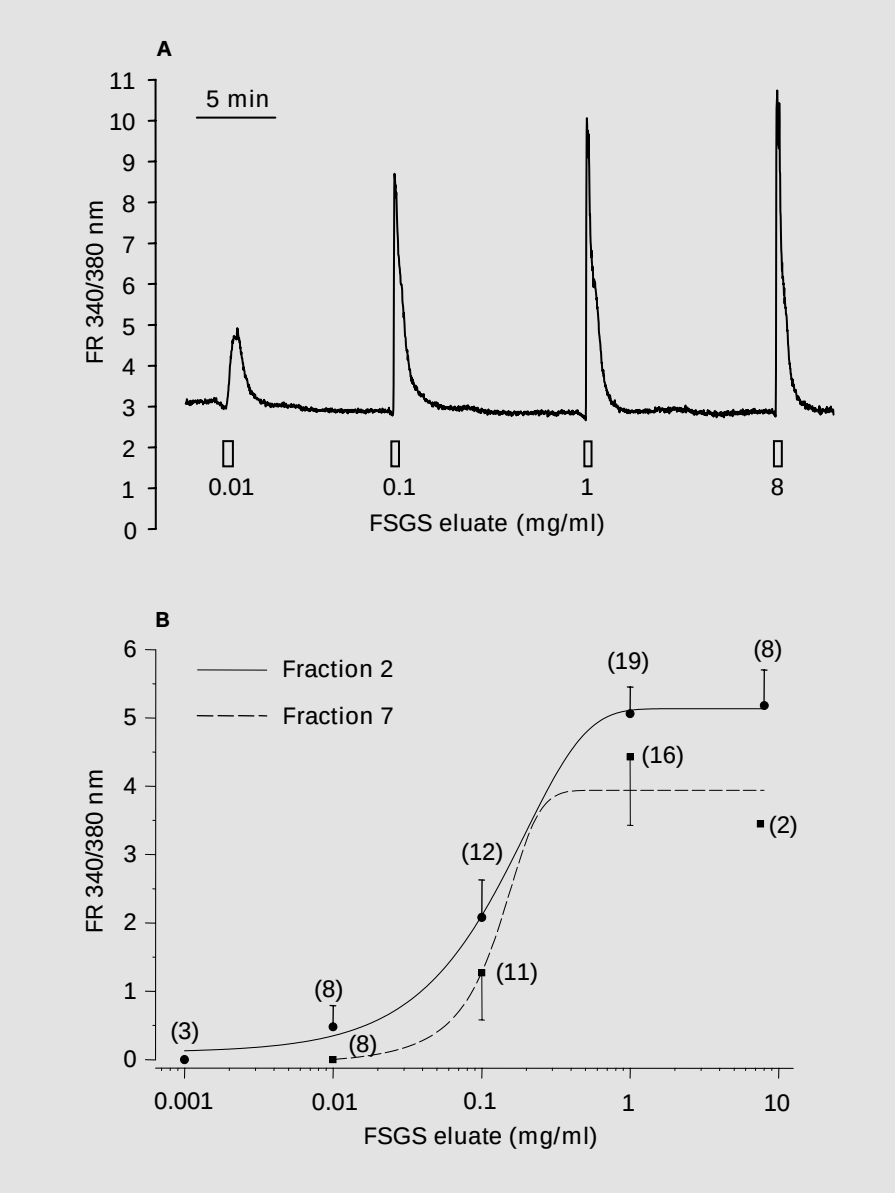

Fig. 1. Eluate derived by IA from a patient suffering from recurrent FSGS elevates the $[Ca^{2+}]_i$ of differentiated podocytes. A Original recording of the $[Ca^{2+}]_i$ response of a single differentiated podocyte to increasing concentrations of FSGS eluate. B Concentration-response curves of different eluate fractions collected either shortly after the start (fraction 2, EC_{50} 0.14 mg/ml) or in the middle (fraction 7, EC_{50} 0.13 mg/ml) of the same IA session, respectively. FR = Fluorescence ratio.

Results

Eluate Derived by IA from an FSGS Patient Elevates the $[Ca^{2+}]_i$ in Differentiated Podocytes

Eluate derived by antibody-based IA from a FSGS patient increased the $[Ca^{2+}]_i$ of differentiated mouse podocytes in a rapid and reversible manner (fig. 1A). This effect was concentration-dependent with an EC_{50} of 0.14 mg/ml (n = 3–19, fig. 1B). 1 mg/ml eluate increased the $[Ca^{2+}]_i$ reversibly from 82 ± 12 to 1,462 ± 370 nmol/l which returned back to 100 ± 16 nmol/l (n = 19). This eluate-induced increase of $[Ca^{2+}]_i$ consisted of an initial Ca^{2+} peak mainly due to release of Ca^{2+} from intracellular Ca^{2+} stores followed by a Ca^{2+} plateau which depended on extracellular Ca^{2+} (fig. 2A, B; n = 8). As compared with the first eluate fraction, the effect of an eluate fraction collected before the end of the same IA session (fraction No. 7) on $[Ca^{2+}]_i$ was slightly diminished (cf. fig. 1A). Eluate derived from a session rinsing blood and plasma compartment with a Ringer solution did not elevate $[Ca^{2+}]_i$ levels of podocytes (data not shown). Nicardipine, a blocker of L-type Ca^{2+} channels, was without effect on the initial Ca^{2+} spike and the sustained Ca^{2+} plateau (fig. 2A, C; n = 5).

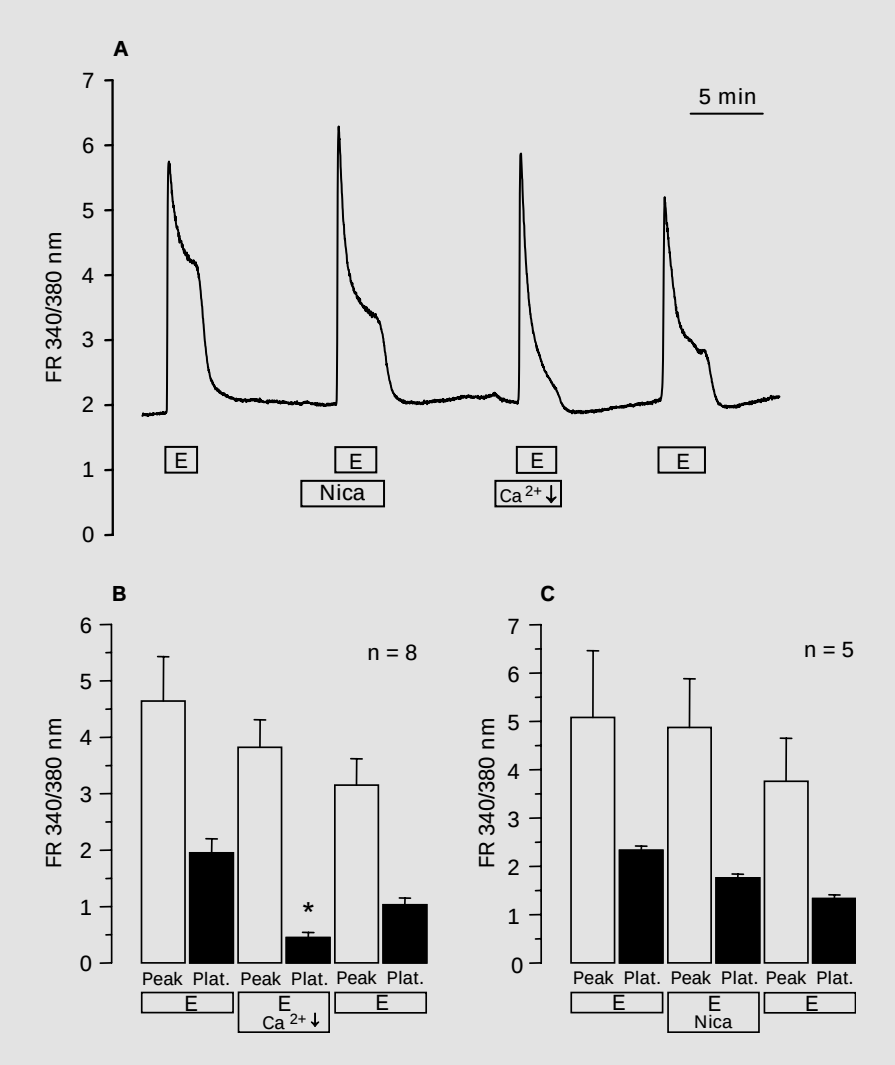

Fig. 2. The FSGS eluate induced Ca^{2+} transient is biphasic with an initial Ca^{2+} peak, followed by a Ca^{2+} plateau, which is dependent on extracellular Ca^{2+}. A Original recording of the effects of a reduced extracellular Ca^{2+} (from 1 mmol/l to 1 µmol/l; $Ca^{2+}\downarrow$) and of nicardipine (Nica; 1 µmol/l) on the eluate-induced Ca^{2+} transient (eluate = 1 mg/ml; E). B Summary of the data (eluate = 1 mg/ml, E; n = 8; * $p < 0.05$). The L-type Ca^{2+} channel blocker nicardipine does not block the FSGS eluate induced transmembrane Ca^{2+} influx. C Summary of the effect of nicardipine (Nica; 1 µmol/l) on the eluate-induced Ca^{2+} transient (eluate = 1 mg/ml, E; n = 5). FR = Fluorescence ratio; plat. = plateau.

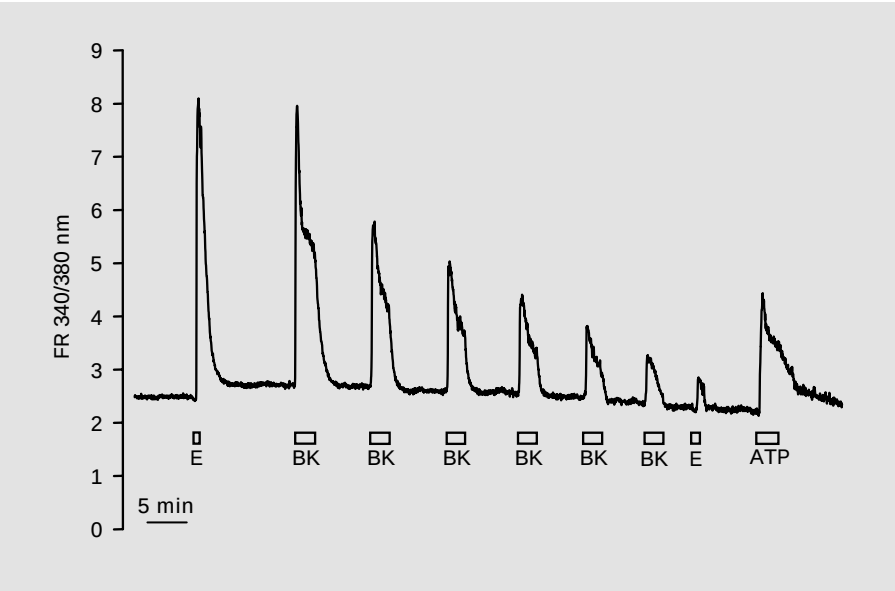

Fig. 3. Repeated application of bradykinin (100 nmol/l; BK) desensitizes the Ca^{2+} response of podocytes to FSGS eluate (1 mg/ml; E). Original recording (n = 5). The response to ATP (100 µmol/l) is not affected. FR = Fluorescence ratio.

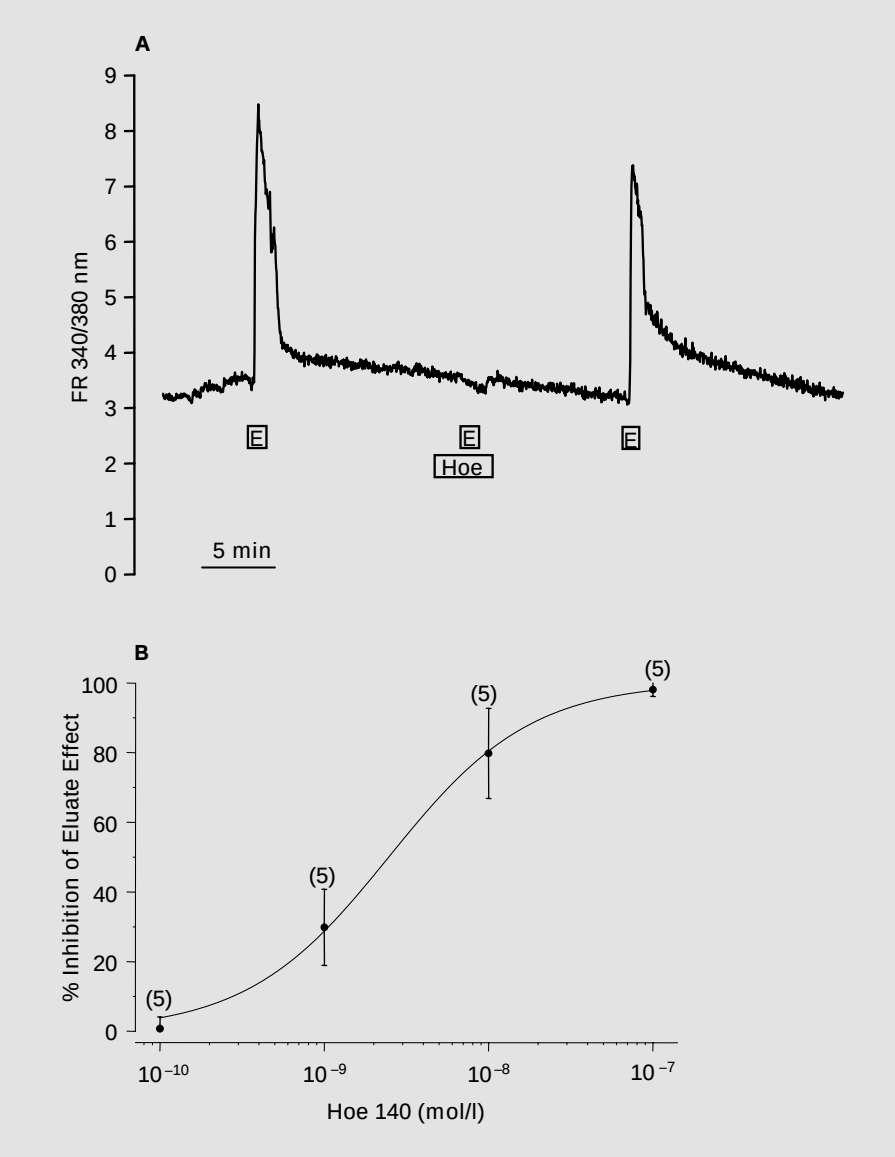

Fig. 4. The FSGS eluate induced Ca^{2+} transient of podocytes is blocked by the specific B_2 kinin receptor antagonist Hoe 140. A Original recording (eluate = 1 mg/ml, E; Hoe 140 = 10 nmol/l, Hoe). B Summary of the data (eluate = 1 mg/ml; IC_{50} 2.47 nmol/l). FR = Fluorescence ratio.

The FSGS Eluate Induced Increase of $[Ca^{2+}]_i$ Is Mediated via a B_2 Kinin Receptor

Repeated application of bradykinin at a concentration of 100 nmol/l led to a decline in the $[Ca^{2+}]_i$ response of podocytes. This effect could not be attributed to a depletion of the internal Ca^{2+} stores, as the time interval between the repeated applications of bradykinin in the presence of 1 mmol/l Ca^{2+} in the bath solution allowed for complete refilling of Ca^{2+} stores. Application of eluate (1 mg/ml) following these repeated bradykinin pulses led to a markedly diminished Ca^{2+} response of podocytes (n = 5). The response to another IP_3-elevating agent, the purinoceptor agonist adenosine triphosphate (ATP; 100 µmol/l), was not affected. Figure 3 depicts an original recording of the effect of eluate (1 mg/ml) before and after repeated bradykinin (100 nmol/l) pulses.

The specific B_2 kinin receptor antagonist Hoe 140 reversibly inhibited the eluate-induced elevation of $[Ca^{2+}]_i$ in a concentration-dependent manner. Figure 4A shows an original recording of the effect of Hoe 140 (10 nmol/l) on the eluate (1 mg/ml) induced Ca^{2+} transient; figure 4B summarizes the data (IC_{50} 2.47 nmol/l; n = 5 for each data point). As blockade of bradykinin receptors by Hoe 140 completely abolished the Ca^{2+} response to eluate, the Ca^{2+}-liberating activity in eluate thus only was by kinin.

Table 1. Free kinins in eluates derived by antibody-based IA

Incubation period min	2 mmol/l EDTA (+/–)	FSGS eluate				Control eluate			
		without podocytes	n	with podocytes	n	without podocytes	n	with podocytes	n
4	–	1,533 ± 79	3	2,100 ± 258	3	3,080 ± 223	3	2,507 ± 111	3
4	+	1,459[a]	2	1,459 ± 179	3	3,710[a]	1	3,107 ± 162	3
40	–	2,288 ± 242	3	1,711 ± 192	3	4,503 ± 290	3	2,413 ± 210	3
40	+	2,520 ± 252	3	1,831 ± 364	3	4,020 ± 79	3	4,167 ± 313	3

Eluates were dissolved in a Ringer-like solution with or without 2 mmol/l EDTA, followed by incubation for the time periods indicated either with or without podocytes. Preparation of aliquots for kinin measurements was performed as described in Subjects and Methods. Kinin was measured by RIA (mean ± SEM; n = number of measurements); the kinin concentration is given in picomoles per liter.

[a] Accidentally not performed in triplicate.

The concentration-response curve of the FSGS eluate resembles that of bradykinin published recently by us [18] for the same cell preparation used in the experiments shown here. The Ca^{2+} increase induced by an eluate concentration of 0.2 mg/ml was approximately equivalent to that due to 0.5 nmol/l bradykinin.

FSGS Eluate Does Not Elevate $[Ca^{2+}]_i$ in Cells Unreactive to Bradykinin

A human epithelial cell preparation derived from a colon carcinoma (HT_{29}) has been recently described not to react to bradykinin by an increase of $[Ca^{2+}]_i$, even if applied at unphysiologically high concentrations [28]. To test for reactivity to eluate, additional microfluorescence experiments were performed with this cell line. Neither bradykinin (1 µmol/l) nor eluate (1 mg/ml) induced an increase of $[Ca^{2+}]_i$. In contrast, the response to ATP (10 µmol/l), which is known to increase $[Ca^{2+}]_i$ in HT_{29} cells [28, 29], was not affected (n = 6, data not shown).

The Eluate-Induced Elevation of $[Ca^{2+}]_i$ Is Caused by Free Kinin

As the FSGS eluate induced $[Ca^{2+}]_i$ increase could be blocked by the specific B_2 kinin receptor antagonist Hoe 140, it seemed likely that this effect was explained by free kinins in the eluate preparation. Thus measurements of kinins in different eluate preparations were performed. We also studied whether kinin-releasing or kinin-degrading enzymes were present in the eluates or at the outer cell membrane or were released from podocytes.

FSGS eluates derived by IA contained kinin in the picomolar range (table 1). In most of the experiments kinin concentrations were increased after a longer incubation period (40 vs. 4 min), indicating that a low kininogenase activity was present. Incubations in the presence of EDTA (2 mmol/l), an inhibitor of kininases, did not cause an alteration of the kinin concentration, demonstrating the absence of active kininases. Thus, the $[Ca^{2+}]_i$ increase was obviously caused by the free kinin present in the eluate preparations. No significant change of the kinin concentration was observed during incubation of eluates with podocytes.

Free Kinin in Eluate Derived by Antibody-Based IA Is Not Specific for FSGS

To test for the specificity of kinin in the IA eluate for FSGS, in a different series, eluate of a healthy volunteer was used. This eluate was prepared exactly in the same way as that of the FSGS patient. Control eluate increased $[Ca^{2+}]_i$ of podocytes in a fashion similar to that of FSGS eluate. Figure 5 shows the respective concentration-response curve (n = 3–8). As compared with the FSGS eluate, the $[Ca^{2+}]_i$ increase induced by control eluate at the same concentration seemed more pronounced which was also reflected in a certain shift of the concentration-response curve (EC_{50} 0.05 mg/ml; cf. fig. 5). The $[Ca^{2+}]_i$ increase induced by control eluate again could be reversibly blocked by Hoe 140 (10 nmol/l; n = 5, data not shown). Control eluates derived by IA contained kinin in the picomolar range (table 1). As compared with the FSGS eluate, the kinin levels in the control eluates were increased. Like with FSGS eluates, the kinin concentrations in the control eluates were increased after a longer incubation period (40 vs. 4 min).

Eluate derived in the same way from a patient suffering from SLE with nephrotic proteinuria likewise elevated

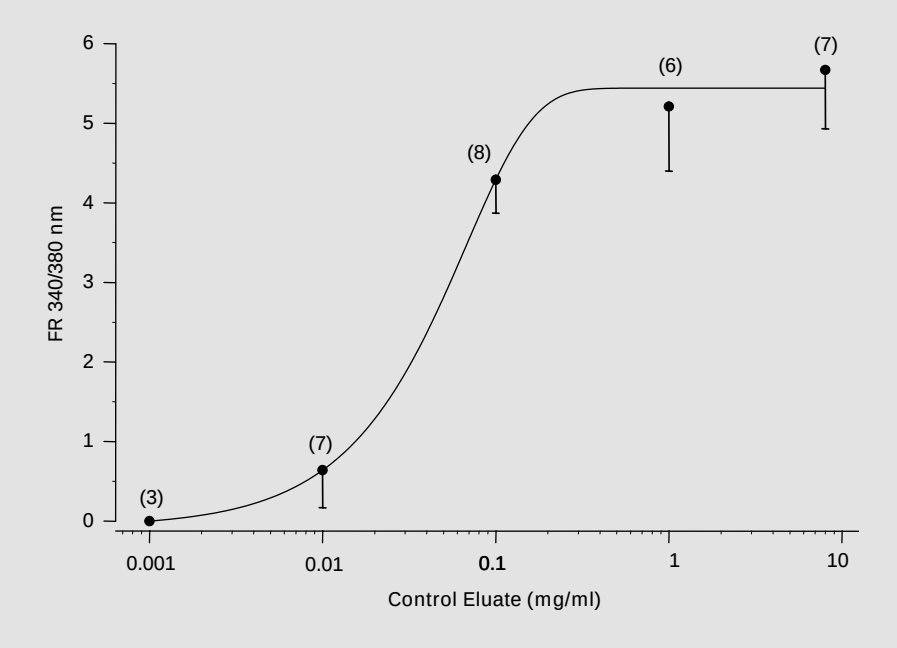

Fig. 5. Eluate from a healthy volunteer elevates the $[Ca^{2+}]_i$ of podocytes. Concentration-response curve (EC_{50} 0.05 mg/ml). FR = Fluorescence ratio.

$[Ca^{2+}]_i$ of podocytes (n = 5, data not shown). The respective $[Ca^{2+}]_i$ increase could also be reversibly blocked by Hoe 140 (10 nmol/l; n = 5, data not shown).

Discussion

In FSGS, IA either using a Sepharose-bound *Staphylococcus* protein A [30] or a Sepharose-bound sheep antihuman immunoglobulin preparation [31] primarily aimed at removing immunoglobulins. Despite of that, at present, it seems unlikely that intact immunoglobulins are directly causing the disease, as the approximate molecular weight of the incriminated factor(s) was below 100 kD [5]. Based on the specificity of IA for binding immunoglobulins, Dantal et al. [32] hypothesized that immunoglobulins are directly or indirectly involved in the mechanism leading to proteinuria in FSGS patients. Whether immunoglobulin fragments are involved, or proteins bound to immunoglobulins, is not clear at present. Both IA procedures were reported to result in the removal of the same putative albuminuric factor which altered the glomerular albumin permselectivity of purified glomeruli in vitro [32]. In contrast to studies showing the antiproteinuric effects of IA procedures to be specific for FSGS, Esnault et al. [11] demonstrated that IA also efficiently reduced proteinuria caused by a number of other diseases: membranous nephropathy, diabetes mellitus, IgA nephropathy, and amyloidosis. Based on their data, they suggested that IA removes a nonspecific circulating hemodynamics-altering or permeability-increasing factor [11].

Podocytes are highly specialized cells which form the outer part of the glomerular filtration barrier, contributing to its specific size and charge characteristics. With regard to FSGS, an early injury of podocytes has been assumed [33]. This led to the description of the initial phase of FSGS in man as 'podocytosis' [34]. In the present study, we investigated the Ca^{2+} response of cultured, differentiated mouse podocytes to eluate derived by extracorporal antibody-based IA both from a patient with recurrent FSGS and a healthy individual. Likewise, the eluate from a patient with SLE suffering from nephrotic syndrome owing to lupus nephritis in her renal transplant was investigated. All three eluates markedly elevated the $[Ca^{2+}]_i$ level of podocytes. This Ca^{2+} increase was rapid and reversible and consisted of an initial peak due to release of Ca^{2+} from intracellular stores and a second plateau-like phase due to influx of Ca^{2+} from the extracellular space. The FSGS eluate induced Ca^{2+} increase was concentration dependent (EC_{50} 0.14 mg/ml). A number of observations provide evidence that this Ca^{2+} increase is due to activation of B_2 kinin receptors which are known to be expressed on the surface of podocytes [35, 36]: (1) The concentration-response curve of eluate resembles that of bradykinin recently published by us for the same cell preparation [18]. (2) Repeated application of bradykinin

at a concentration of 100 nmol/l desensitized B_2 kinin receptors. The Ca^{2+} response to FSGS eluate following these bradykinin pulses was markedly reduced. (3) The specific B_2 kinin antagonist Hoe 140 [37] reversibly inhibited the eluate-induced elevation of $[Ca^{2+}]_i$. (4) Cells not expressing B_2 kinin receptors did not respond to eluate with an increase of $[Ca^{2+}]_i$. Furthermore, kinins were detected in the eluate preparation by radioimmunoassay. As compared with the short half-life of kinins in the circulation, due to degradation, e.g., by kininases, kinins in eluate were stable and did not diminish over time. On the contrary, the kinin concentrations increased by prolonged incubation which may be attributed to an ongoing release of kinins from high molecular weight precursors. In this regard, the eluates used in this study contained relevant amounts of (trypsin-)releasable kinins.

B_2 kinin receptors have been described in numerous tissues of different species [38]. Their activation among other effects uniformly leads to an inositol phosphate mediated increase of $[Ca^{2+}]_i$, which can be prevented by the potent selective B_2 kinin receptor antagonist Hoe 140 [37, 38]. Within this study, differentiated human podocytes were not available. Therefore, experiments were performed with differentiated mouse podocytes which showed an arborized morphology and expression of the podocyte markers WT-1 [15], synaptopodin [19], nephrin [20], p57 [21], and CD2AP [22]. With B_2 kinin receptors already having been demonstrated in nondifferentiated human podocytes [35, 36], we believe that similar results are to be expected upon investigation of differentiated human podocytes.

As antibody-based IA specifically removes immunoglobulins from plasma, at first it is surprising that the eluates under investigation contained significant amounts of free kinin. The source of these kinins is not clear yet. Kinins may be generated by the IA procedure itself; generation of bradykinin has been described in low-density lipoprotein (LDL) adsorption due to use of the negatively charged dextran sulfate cellulose [39] as well as in hemodialysis with negatively charged dialyzer membranes such as polyacrylonitrile, especially in combination with ACE inhibitors [40]. Whether kinin is generated within the setup used in this study, mainly consisting of a large-pore plasmapheresis filter and Sepharose-containing IA columns, is not known at present. Using a dextran sulfate cellulose membrane for LDL apheresis, Murashima et al. [39] demonstrated a marked depletion of high molecular weight kininogen and prekallikrein on the one hand and an increase of the bradykinin concentration on the other. In addition, LDL apheresis has been described to decrease proteinuria in patients with minimal change nephrotic syndrome and membranous nephropathy [41]. Based on these observations, Esnault et al. [11] suggested that the hemodynamics-altering factor mentioned above could possibly be removed both by IA and LDL apheresis [11].

With regard to proteinuria, the role of kinins has not yet been fully elucidated. Inhibition of ACE (also known as kininase II) does not only reduce angiotensin II, but also decreases the breakdown, e.g., of bradykinin with subsequent elevation of kinin levels. For this reason, it has been a matter of debate whether the well-established antiproteinuric effect of ACE inhibition is due to a reduction of intrarenal angiotensin II or to a decreased bradykinin degeneration [42]. In vivo studies on this item provided conflicting results; some found a significant contribution of bradykinin to the antiproteinuric effect of ACE inhibitors [43], others did not [42]. Concerning permeability plasma factors, it appears questionable whether a single unique mechanism leads to nephrotic proteinuria [10]. A number of diverse factors may contribute to its pathophysiology which could work as initiators, amplifiers, or late effectors, as discussed by Esnault et al. [11]. In this regard, the role of kinins awaits further clarification.

In summary, we have shown for the first time that eluate derived by antibody-based IA contains high amounts of free kinins, affecting cellular functions of podocytes by activating B_2 kinin receptors. As podocytes are the primary target of injury in proteinuric diseases, here the role of kinins deserves further investigation. It should be noted, however, that raw eluate may contain a diversity of biologically active substances. Hence, with regard to causal implications for the pathogenesis of proteinuric diseases, results directly obtained with eluate from IA procedures should be interpreted with appropriate care.

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