Low extracellular Ca\(^{2+}\): a mediator of endothelial inflammation

Yeela Talmor-Barkan\(^{1,2}\), Gloria Rashid\(^1\), Iris Weintal\(^1\), Janice Green\(^1\), Jacques Bernheim\(^{1,2}\) and Sydney Benchetrit\(^1\)

\(^{1}\)Renal Physiology Laboratory, Department of Nephrology and Hypertension, Meir Medical Center, Kfar-Saba and \(^2\)Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

**Abstract**

**Background.** Recent studies have suggested that vitamin D and an imbalance in calcium homeostasis may have an impact on the cardiovascular system. The aim of this study was to assess the impact of different concentrations of extracellular Ca\(^{2+}\) on human umbilical vein cord endothelial cells (HUVEC) by measuring its effect on parameters involved in the pathogenesis of vascular inflammatory responses.

**Methods.** HUVEC were grown in the 3.5, 4.5 or 5.8 mg/dL concentration of extracellular Ca\(^{2+}\) for 2–3 weeks. Expression of adhesion molecules was analysed by flow cytometry. Endothelial nitric oxide synthase (eNOS), receptor of advanced glycation end-product (RAGE) and interleukin-6 (IL-6) mRNA expressions were determined by real-time PCR. eNOS, inhibitor kappa B\(\zeta\) (I\(\kappa\)B\(\zeta\)) and phosphorylated I\(\kappa\)B\(\alpha\) protein levels by Western blot, eNOS activity by conversion of \(^{14}\)C-arginine to \(^{14}\)C-citrulline, IL-6 and osteoprotegerin (OPG) secretion by ELISA and DNA-binding activity of nuclear factor kappa B (NF\(\kappa\)B)-p65 were assayed colorimetrically in nuclear extracts.

**Results.** In the presence of low Ca\(^{2+}\) (3.5 mg/dL), protein expressions and activity of eNOS were diminished, while the protein expressions of intercellular adhesion molecule-1 (ICAM-1), as well as the mRNA expressions of RAGE and IL-6, were elevated. The protein secretions of IL-6 and OPG were also stimulated in low Ca\(^{2+}\) concentration. At this concentration, the DNA-binding activity of NF\(\kappa\)B was enhanced, probably due to the decreased level of I\(\kappa\)B\(\alpha\).

**Conclusions.** These results suggest that lower extracellular ionized Ca\(^{2+}\) may play a relevant role in modifying endothelial cells functions.

**Keywords:** calcium; endothelial cells; NF\(\kappa\)B

---

**Introduction**

Cardiovascular diseases remain the main cause of dialysis-related morbidity and mortality [1]. Disturbances in calcium regulatory hormones and high levels of phosphate and calcium phosphate products in chronic kidney disease (CKD) are associated with increased morbidity and mortality, mostly due to cardiovascular complications [2]. A possible relationship between extracellular ionized calcium and the cardiovascular system has been previously suggested. It was found that the calcium intake in women with preeclampsia is significantly lower compared to that in healthy pregnant women. In addition, a risk reduction in pregnancy-induced hypertension and preeclampsia was observed while taking calcium supplements [3]. Such an effect could be explained by the well-known action of physiological concentrations of Ca\(^{2+}\) on the endothelial synthesis of nitric oxide (NO) [4]. Previous clinical studies have demonstrated that low blood levels of calcium are associated with increased mortality [5]. Most patients with progressive chronic renal failure develop impaired homeostasis of extracellular ionized calcium as a result of decreased renal production of calcitriol and increased blood levels of phosphorus [6,7]. This condition is associated with the accelerated development of vasculopathies [8]. Nevertheless, many questions concerning the possible direct impact of low extracellular calcium concentrations on vascular cells and consequently on vascular homeostasis remain unanswered. Extracellular calcium regulates the functions of many cell types through a G-protein-sensing receptor [9]. Therefore, the detection of functional extracellular calcium-sensing receptors (CaR) in human endothelial cells could suggest such impact [10,11].

Based on the preceding findings, we used an in vitro model to examine whether changes in extracellular Ca\(^{2+}\) concentrations affect the function of endothelial cells. In the presence of different concentrations of extracellular Ca\(^{2+}\), we examined the expressions, secretions and activities of well-recognized pro-inflammatory/atherosclerotic parameters, including endothelial nitric oxide synthase (eNOS), intercellular adhesion molecule-1 (ICAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1), receptor of advanced glycation end-products (RAGE), interleukin-6 (IL-6) and osteoprotegerin (OPG). In addition, the activation of the nuclear factor kappa B (NF\(\kappa\)B) pathway has been determined by knowing its involvement in endothelial inflammatory responses [12].
Subjects and methods

Endothelial cell culture and incubation

Endothelial cell cultures were obtained from human umbilical vein endothelial cells (HUVEC) as previously described [13]. The ethical committee of Meir Medical Center approved the research programme, and all parturients gave written consent permitting the use of the umbilical cord. The cells were identified as endothelial cells by their typical cobblestone morphology and by immunostaining for von Willebrand factor. HUVEC were grown under standard cell culture conditions (humidified atmosphere, 5% CO₂, 37°C) in a M-199 medium containing 20% fetal calf serum (FCS), 100 μg/mL penicillin, 100 μg/mL streptomycin (Biological industries, Israel), 0.25 μg/mL amphotericin, 5 units/mL Heparin and 25 μg/mL endothelial mitogen (Biomedical technologies Inc., USA). The final Ca²⁺ concentration of 3.5, 4.5 or 5.8 mg/dL was measured by Radiometer ABL (Copenhagen, Denmark). The cells were grown in different concentrations of extracellular ionized calcium (3.5, 4.5 or 5.8 mg/dL) immediately after obtaining them from umbilical cord (from Day 0) until they were harvested (after 2–3 weeks). The protocol of 2–3 weeks' incubation time was based on the concept that the prolonged low Ca²⁺ concentration is present in CKD patients. The cells were cultured through three to four passages under the different Ca²⁺ concentrations. These concentrations were corresponding to physiological and pathophysiological concentrations in human sera. The concentration of 3.5 mg/dL was corresponding to low values, 4.5 mg/dL was considered as normal level and 5.8 mg/dL as the standard Ca²⁺ concentration in the milieu of cell culture and an elevated value found in hypercalcaemic conditions observed in clinical settings.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The total RNA was extracted from endothelial cells using the PUREscript RNA isolation kit (Genta Systems, MN, USA), according to the manufacturer's instructions. RNA (1 μg) was then reverse transcribed into single-strand DNA with 200 U of SuperScript II RNase Transcriptase (Invitrogen, CA, USA) and oligo (dT)15 primer (Promega, Madison, WI, USA) at 37°C for 45 min, 42°C for 15 min and 99°C for 5 min.

To quantify the amounts of eNOS, RAGE and IL-6 mRNA expression in endothelial cells, real-time PCR was performed with a LightCycler instrument (Roche Diagnostics GmbH, Mannheim, Germany) in glass capillary tubes. The Light-Cycler Fast Start DNA Master SYBR Green I reaction mix (Roche Diagnostics GmbH) and primers were added to cDNA dilutions. eNOS primers were forward primer 5'-CCCTGACATGAGGATCTG-3', reverse primer 5'-CGTGAGCCAAATGTCTTC-3', RAGE primers were forward primer 5'-TGGACACGCCTAATCACC-3', reverse primer 5'-CGATGTGCTGATGCTGACA-3', IL-6 primers were forward primer 5'-GGTACATCCTCGACGGCCT-3', reverse primer 5'-GGTGGGTACGGGGTTGTATTG-3'. β-Arrestin primer sequences were forward primer 5'-GACACACCTCTAACATGAG-3' and reverse primer 5'-GCTATCCCCGATAGTGGG-3'. The thermal profile for SYBR Green PCRs was 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, 58°C for 7 s, 72°C for 18 s and 90°C for 5 s. To prove the specificity of the PCR product, a melting curve analysis was performed by 95°C for 5 s and 70°C for 20 s. A dilution series of a standard sample was run with the unknown samples. Gene expression was determined by normalization against β-actin expression.

Western blot analysis

To evaluate the protein expression of eNOS, IκBα, phosphorylated IκBα and α-tubulin, total protein (50 μg) was electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk, incubated with an anti-eNOS monoclonal antibody (1:1000; BD Transduction Laboratories, Lexington, KY, USA), anti-IκBα monoclonal antibody (1:500; Santa Cruz, CA, USA), anti-phosphorylated IκBα (p-IκBα) monoclonal antibody (1:400; Santa Cruz) or with an α-tubulin monoclonal antibody (1:13 000; Sigma, MI, USA). The secondary antibody was sheep anti-mouse IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch Labs Inc., West Grove, PA, USA) and developed with the chemiluminescent reporter system. The nitrocellulose membranes were stripped and blocked before being reprobed with a different antibody. The expression of the eNOS protein was detected as a single band at 150 kDa, the expression of IκBα and p-IκBα proteins were detected as a single band at 37 kDa and α-tubulin as the loading control was detected as a single band at 50 kDa.

eNOS activity assay

To measure eNOS activity in cell lysates (150 μg total protein), the conversion of [¹⁴C]-arginine to [¹⁴C]-citrulline was used as previously described by Shah et al. [14].

Flow cytometry analysis

To evaluate the membrane expression of ICAM-1 and PECAM-1 expression, 3 × 10⁶ cells were incubated with 20 μL of the PE-conjugated monoclonal antibody (anti-ICAM-1, anti-PECAM-1; Immunotech, Marseille, France) for 30 min at 4°C. IgG1-matched isotypes were used as the control. After washing, fluorescence was analysed by flow cytometry (FACS) employing a Coulter Flow Cytometer (EPICS-XL, Beckman Coulter, UK). A total of 10 000 events were counted in each FACS analysis. The data represent the mean fluorescence intensity (MFI) (arbitrary units) of the entire population.

IL-6 and OPG immunoassay

IL-6 or OPG was measured in HUVEC supernatants using a DuoSet enzyme-linked immunosorbent assay (ELISA) development kit (R&D Systems, Minneapolis, MN, USA). IL-6 and OPG levels are given as pg/10⁵ cells after correction of cell number.

NfκB DNA-binding activity assay

Nuclear protein extracts were prepared using the NucBuster Protein Extraction Kit (Novagen, Madison, WI, USA) according to the manufacturer's instructions. DNA-binding activity of NfκB was assayed colorimetrically, using NoShift NFκB (p65) reagents and the NoShift Transcription Factor Assay Kit (Novagen). Negative controls consisted of reactions performed in the absence of nuclear extract. As a secondary antibody, we used HRP-conjugated goat anti-mouse IgG that targets the anti-NFκB-p65 mouse monoclonal antibody. All assays were performed in duplicate. Binding activity was measured via colorimetric absorbance at 450 nm on a spectrophotometer (Sunrise, NEOTEC, Grodig, Austria) using 3,3′,5,5′-tetramethylbenzidine as a substrate.

Statistical analysis

The results are expressed as mean ± SE. Student's paired t-test was used for data analysis. P-values of < 0.05 were considered significant.

Results

Effect of extracellular Ca²⁺ on the eNOS system

The cytoplasmic protein expression of eNOS was significantly depressed in a low Ca²⁺ environment (Figure 1A). α-Tubulin was used as the loading control. In addition, the enzymatic activity of eNOS was significantly suppressed (65.9 ± 21%, versus control, P = 0.04; Figure 1B). The eNOS activity was not significantly decreased in the presence of 4.5 mg/dL concentration of extracellular Ca²⁺ (87.5 ± 22% versus control, P > 0.1; Figure 1B). The expression of eNOS mRNA was not affected by the presence of different concentrations of Ca²⁺ (data not shown).

Low extracellular Ca²⁺ increased the expression of endothelial ICAM-1

Low extracellular Ca²⁺ increased the MFI of ICAM-1 compared to the MFI observed in the presence of 5.8 mg/dL Ca²⁺ (119.3 ± 9.2% versus control, P = 0.031; Figure 2). Different concentrations of Ca²⁺ did not affect the number
Fig. 1. Effect of extracellular ionized calcium on the eNOS system. (A and B) HUVEC were grown in different concentrations of extracellular ionized calcium (3.5, 4.5 and 5.8 mg/dL). (A) Protein lysates were prepared. eNOS protein expression was assessed by Western blot analysis. The level of α-tubulin is shown as a loading control. Similar results were obtained in four independent experiments. (B) eNOS activity in total cell lysates was measured by conversion of $[^{14}C]$-arginine to $[^{14}C]$-citrulline. Data are expressed as mean ± SE of six to nine independent experiments. $^*P ≤ 0.04$ versus control (5.8 mg/dL Ca$^{2+}$).

Fig. 2. Effect of extracellular ionized calcium on ICAM-1. HUVEC were grown in different concentrations of extracellular ionized calcium (3.5, 4.5 and 5.8 mg/dL). Fluorescence was analysed by flow cytometry employing a Coulter EPICS-XL Flow Cytometer (FACS). A total of 10 000 events were counted in each FACS analysis. The relative mean fluorescence intensity (MFI) of ICAM-1 was expressed as fold of control values (5.8 mg/dL Ca$^{2+}$). Data are expressed as mean ± SE of six independent experiments. $^*P < 0.05$ versus control (5.8 mg/dL Ca$^{2+}$).

Fig. 3. Effect of extracellular ionized calcium on RAGE expression. HUVEC were grown in different concentrations of extracellular ionized calcium (3.5, 4.5 and 5.8 mg/dL). Total RNA was extracted and the levels of RAGE and β-actin mRNA expressions were assessed by real-time PCR. RAGE mRNA levels normalized to the levels of β-actin mRNA expressions, and relative mRNA content was expressed as fold of control. Data are expressed as mean ± SE of five independent experiments. $^*P ≤ 0.05$ versus control (5.8 mg/dL Ca$^{2+}$).

Fig. 4. Effect of extracellular ionized calcium on IL-6 expression and secretion. (A and B) HUVEC were grown in different concentrations of extracellular ionized calcium (3.5, 4.5 and 5.8 mg/dL). (A) Total RNA was extracted, and the levels of IL-6 and β-actin mRNA expressions were assessed by real-time PCR. IL-6 mRNA levels normalized to the levels of β-actin mRNA expressions and relative mRNA content was expressed as fold of control. Data are expressed as mean ± SE of five independent experiments. $^*P ≤ 0.04$ versus control (5.8 mg/dL Ca$^{2+}$). (B) IL-6 in supernatants was measured by ELISA. Results are mean ± SE of three to four independent experiments. $^*P < 0.05$ versus control.

Effect of extracellular Ca$^{2+}$ on RAGE mRNA

The expression of RAGE mRNA was increased in the presence of a low Ca$^{2+}$ concentration of 3.5 mg/dL (159.3 ± 15.3% versus control, $P = 0.018$; Figure 3). No changes were observed when the calcium concentration was 4.5 mg/dL (101.8 ± 29% versus control, $P > 0.1$; Figure 3).

Effect of extracellular Ca$^{2+}$ on IL-6 mRNA expression and protein secretion

In the presence of a low Ca$^{2+}$ concentration, the mRNA expression of IL-6 was significantly stimulated (177 ± 34.5% versus control, $P = 0.044$; Figure 4A). In the presence of a 4.5 mg/dL Ca$^{2+}$ concentration, IL-6 mRNA expression was not significantly elevated (137 ± 51.4% versus control, $P > 0.1$; Figure 4A).
The endothelial secretion of IL-6 was significantly increased in the presence of a low Ca\(^{2+}\) concentration (1550.4 ± 155 pg/10\(^5\) cells versus control: 553.5 ± 98 pg/10\(^5\) cells, \(P = 0.03\); Figure 5) but not in the presence of a 4.5 mg/dL Ca\(^{2+}\) concentration (1167.3 ± 233 pg/10\(^5\) cells versus control: 553.5 ± 98 pg/10\(^5\) cells, \(P = 0.068\); Figure 5).

**Effect of extracellular Ca\(^{2+}\) on OPG protein secretion**

OPG secretion was significantly increased in the presence of low Ca\(^{2+}\) (931 ± 149 pg/10\(^5\) cells versus control 93 ± 8.14 pg/10\(^5\) cells, \(P = 0.03\); Figure 5). In the presence of 4.5 mg/dL Ca\(^{2+}\), the elevation was not significant (332.8 ± 164.7 pg/10\(^5\) cells versus control: 93 ± 8.14 pg/10\(^5\) cells, \(P > 0.1\); Figure 5).

**Low extracellular Ca\(^{2+}\)-induced activation of the endothelial NF\(\kappa\)B pathway**

A low Ca\(^{2+}\) concentration induced a significant increase in the NF\(\kappa\)B-p65 DNA-binding activity (114.44 ± 3.88\% versus control, \(P = 0.032\); Figure 6A), a phenomenon that may be explained by the decreased levels of I\(\kappa\)Ba (Figure 6B). The depressed expression of I\(\kappa\)Ba may result from a decreased synthesis rather than an increased phosphorylation and degradation, as low Ca\(^{2+}\) did not modify the phosphorylation of I\(\kappa\)Ba (Figure 6B). \(\alpha\)-Tubulin was used as the loading control (Figure 6B).

**Discussion**

Cardiovascular complications are the most frequent cause of morbidity and mortality in patients with advanced and end-stage renal failure (ESRF). These complications are frequently associated with disturbances in mineral and bone metabolism characterized by the presence of low blood levels of extracellular Ca\(^{2+}\) and calcitriol and elevated values of PTH [2]. In fact, the occurrence of hypocalcaemia can be detected in clinical conditions that are associated either with low levels of PTH ‘hypoparathyroidism’ or with high blood values of PTH ‘secondary hyperparathyroidism’ (HPT) [15]. The development of secondary HPT is related to a metabolic condition characterized by a low concentration of ionized calcium in the blood as seen, for example, in patients with advanced renal failure. In such situations, there is an increased frequency and severity of cardiovascular diseases, which results from accelerated vascular atherosclerosis, and/or vascular calcifications [16–18].

The effects of calcium regulatory hormones on the vascular system have been well described [19–26]. However, the impact of extracellular Ca\(^{2+}\) itself on the development of cardiovascular complications has not been thoroughly studied. Our present study was conducted to evaluate a possible direct effect of extracellular Ca\(^{2+}\) on endothelial cell functions. Three concentrations of extracellular Ca\(^{2+}\) were used, 5.8 mg/dL, the concentration of Ca\(^{2+}\) in the standard medium of cell cultures, 4.5 mg/dL, a normal Ca\(^{2+}\) concentration in the blood, and 3.5 mg/dL, a low level of extracellular Ca\(^{2+}\). The cells grown in a standard medium of 5.8 mg/dL Ca\(^{2+}\) were referred to as control.

All experiments performed with 5.8 mg/dL and 4.5 mg/dL Ca\(^{2+}\) concentrations did not reveal significant differences. This observation is important since the standard medium for cell cultures contains high concentration of ionized calcium (5.8 mg/dL), while the physiological concentration in blood is lower (such as 4.5 mg/dL). Therefore, we can assume that by using the standard medium, we are not affecting the results of the studied parameters. In the presence of low Ca\(^{2+}\) (3.5 mg/dL), significant endothelial dysfunctions were detected, including an up-regulation of the endothelial NF\(\kappa\)B activity. Knowing its importance in the control of local inflammation [12], the activation of this pathway may explain at least in part why the expressions/secretions of ICAM-1, RAGE, IL-6 and OPG were stimulated and why the eNOS system was depressed.

Previous studies have suggested that extracellular calcium may affect the normal functioning of endothelial cells, and may play a crucial role in the control of NO production and vascular tone [4]. This effect may be regulated by the
CaR expressed on endothelial cell membranes [10,11]. In the present study, we found a significant decrease in eNOS protein expression and activity in a low Ca\(^{2+}\) environment. The depressed activity of the eNOS system suggests a reduction in NO release and may consequently induce platelet aggregation and white blood cell adherence, and stimulate intravascular coagulation [27–29].

In addition, a low Ca\(^{2+}\) environment was associated with increased endothelial expression of the adhesion molecule ICAM-1 that is involved in the cellular interaction of endothelial cells-leukocytes and platelets. Activation and recruitment of circulating blood cells are implicated in the pathogenesis of atherosclerosis [30]. Low Ca\(^{2+}\) was also found to stimulate the expression of RAGE as well as the expression and secretion of IL-6. These two pro-inflammatory parameters are thought to participate in the occurrence of vascular atherosclerosis and inflammation [31–35], particularly when production of advanced glycation end-products (AGEs) is augmented [36]. In fact, RAGE mediates important cellular effects of AGEs, and its expression is markedly elevated in atherosclerotic and other vascular diseases [34,37].

OPG, a soluble receptor that belongs to the tumour necrosis factor receptor superfamily [38] serves as a decoy receptor by binding to a receptor activator of NFκB ligand (RANKL). By blocking the interaction of RANKL with its receptor [39], OPG protects from bone loss [40]. Recently, it was found that circulating OPG may also play a role in cardiovascular homeostasis, as disturbances in the OPG/RANKL system have been implicated in vascular diseases [41,42]. OPG was also identified as a novel marker for cardiovascular mortality in patients with acute myocardial infarction and heart failure [43]. In addition, haemodialyzed patients who suffered from increased vascular calcification and severe coronary artery disease were characterized by increased levels of OPG [44–46]. However, animal studies have shown that OPG deficient mice developed severe medial aortic and renal artery calcification [47], and that administration of OPG was able to prevent vascular calcification induced by warfarin and vitamin D in rats [48]. Therefore, the eventual impact of OPG on vascular homeostasis remains undetermined. OPG produced by endothelial cells is up-regulated by inflammatory mediators and down-regulated by PTH [49,50]. As demonstrated here, low extracellular Ca\(^{2+}\) stimulates the endothelial secretion of OPG, a finding that fits well with the effect of low Ca\(^{2+}\) on the endothelial pro-inflammatory parameters. This increase in OPG secretion should be further investigated in vivo in order to explain, at least in part, why elevated blood values of OPG may be detected in patients with advanced renal failure who are particularly susceptible to developing frequent and severe vasculopathies [44–46].

As mentioned previously, the endothelial inflammatory processes are mainly regulated by the NFκB pathway [12,51,52]. A low Ca\(^{2+}\) environment enhanced the nuclear p65-DNA-binding activity. This effect was associated with depressed expression of IkBα, the main inhibitor of NFκB activity [12]. As the expression of phosphorylated IkBα was not modified, we can assume that the change in IkBα was not due to enhanced phosphorylation and degradation, but rather from a decrease in its synthesis.

We are not aware of other studies dealing with the stimulation of endothelial inflammation by low extracellular Ca\(^{2+}\). We can mention that previous studies did evaluate the effect of different calcium concentrations in preservation solution on vascular tissue [53–56]. The conditions of these studies are not comparable to our in vitro model of EC cultures, which do not deal with whole organ behaviour. In addition, the very low temperature (4°C) used for organ preservation may impair the release of endothelium-dependent relaxing factor [57] and may induce structural EC changes [58]. In our system, the temperature used for standard cell culture condition was 37°C.

The results of the present study may contribute to a better understanding of why and how hypocalcaemia may have a long-term deleterious effect on vascular functions and structures; however, it is clear that cultured EC may show a different behaviour than EC in vivo, at least partly, due to their interaction with the underlying smooth muscle cells. It will be relevant to eventually confirm in vivo the importance of the present results.

References

7. Chan CT, Li SH, Verma S. Nocturnal hemodialysis is associated with reduced NO production by eNOS. Semin Perinatol 2005; 29: 254–260
40. Lacey DL, Timms E, Tan HL et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998; 93: 165–176
45. Jono S, Ikari Y, Shioi A et al. Serum osteoprotegerin levels are associated with the presence and severity of coronary artery disease. Circulation 2002; 106: 1192–1194
Double-stranded RNA activates type I interferon secretion in glomerular endothelial cells via retinoic acid-inducible gene (RIG)-1

Holger Hägele¹, Ramanjaneyulu Allam¹, Rahul D. Pawar¹,² and Hans-Joachim Anders¹

¹Department of Nephrology, Medical Policlinic, University of Munich, Munich, Germany and ²Division of Rheumatology, Albert Einstein College of Medicine, Jack and Pearl Resnick Campus, Bronx, NY 10461, USA

Correspondence and offprint requests to: Hans-Joachim Anders; E-mail: hjanders@med.uni-muenchen.de

Abstract

Background. The molecular pathomechanisms by which viral infections trigger glomerulonephritis remain elusive. In the glomerulus, glomerular endothelial cells (GEnC) first interact with circulating viral particles; hence, we hypothesized that viral RNA, a known inducer of type I interferons and cytokines in dendritic cells, would also elicit proinflammatory antiviral responses in GEnC.

Methods. Cultured murine GEnC were stimulated with poly I:C RNA and phenotype changes were assessed. Specific antagonists or siRNA were used to determine the mechanisms of RNA uptake and the functional role of putative RNA receptors.

Results. Poly I:C RNA activated GEnC to produce IL-6, CCL2, CCL5, CXCL10, IFN-α and IFN-β. This was independent of endosomal acidification or MyD88 but required complex formation with cationic lipids to be taken up into GEnC via clathrin-dependent endocytosis. RIG-1-dependent complex formation with cationic lipids to be taken up into GEnC via clathrin-dependent endocytosis. RIG-1 but not MDA5-specific s.i.RNA prevented GEnC activation. Type I interferon production did not activate GEnC in an autocrine-paracrine manner. Complexed RNA also activated GEnC to express ICAM-1 and increased the albumin permeability of GEnC monolayers.

Conclusions. Complexed dsRNA enters GEnC via clathrin endocytosis and activates GEnC via RIG-1 in the cytosol to produce inflammatory cytokines, chemokines and type I interferons. Furthermore, RNA induces ICAM-1 expression and increases GEnC permeability. All of these mechanisms may contribute to the onset or aggravation of glomerulonephritis associated with RNA virus infections.

Keywords: endothelial cells; glomerulonephritis; inflammation; interferon; viral infection

Introduction

Extrarenal RNA virus infections can trigger de novo immune complex glomerulonephritis, e.g. hepatitis C virus-associated glomerulonephritis, but more frequently RNA viruses trigger disease activity of preexisting glomerular inflammation, like in IgA nephropathy, lupus nephritis or renal vasculitis [1]. Viral infection activates systemic antiviral immune responses that may trigger disease flares of glomerulonephritis by enhancing autoantibody production, immune complex formation or by systemic interferon (IFN) release [2]. Induction of type I IFN is a central element of antiviral immunity because type I IFNs inhibit viral replication in the infected cells and have pleiotrophic immunomodulatory effects [3]. In the intravascular compartment, plasmacytoid dendritic cells (pDC) are the main type I IFN-producing cell type [2]. Single- and double-stranded RNA activate type I IFN production only after being taken up into intracellular endosomes where they activate TLR3 (dsRNA) or TLR7/8 (ssRNA) [4,5]. Furthermore, viral RNA entering the intracellular cytosol of pDCs triggers type I IFN production via two helicases, i.e. melanoma-differentiation-associated gene (MDA)-5 (dsRNA) and retinoic-acid-inducible protein (RIG)-1 (5′-triphosphate RNA) [6–10].

Despite the obvious clinical association of viral infection and glomerulonephritis, it is unknown whether renal cells produce type I IFN in glomerulonephritis. We recently observed that viral dsRNA can activate murine mesangial cells to produce large amounts of IFN-α and IFN-β when the RNA reaches the intracellular cytosol where it activates TLR-independent RNA recognition pathway ([Flt1r, et al., under revision]). Most remarkably, the type I IFN...