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Jacobs Journal of Nephrology and Urology

Research Article

Uremic Toxins Deregulate the Expression of miR-29a and miR-29b in Endothelial Cells.

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Received: 07-11-2014

Accepted: 09-12-2014

Published: 11-04-2014

Abstract

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in patients with chronic kidney disease (CKD). The uremic toxins that accumulate in CKD patients as a result of impaired kidney function are involved in vascular dysfunction and increase the already high risk of cardiovascular mortality in this population. Although recent studies suggest that dysregulation of the microRNA (miRNA)-29 family is associated with impaired vascular function, the effect of uremia on miR-29 expression in the vascular wall has yet to be characterized. To this end, we investigated the effect of uremic serum and two representative uremic toxins (inorganic phosphate (Pi) and indoxyl sulfate (IS)) on miR-29a and miR-29b expression in human umbilical vein endothelial cells (HUVECs). After 24 h and five days of treatment with uremic serum, there were no significant differences in miR-29a and miR-29b expression (relative to control experiments with non-uremic serum). In HUVECs, 24 h (but not five days) of incubation with IS was associated with downregulation of miR-29a and miR-29b expression. In HUVECs, five days (but not 24 h) of incubation with Pisignificantly increased the expression of both miR-29a and miR-29b. We also found those 10 weeks of CKD upregulatesmiR-29a and miR-29b in the murine aorta. Overall, our results suggest that the various uremic toxins in uremic serum (including Piand IS) are associated with both upregulation and downregulation of miR-29s in vascular walls - emphasizing the complexity of miR-29 regulation in CKD.

Keywords: Uremic Toxin; Indoxyl Sulfate; Endothelial Cell; Chronic Kidney Disease

Abbreviations

CVD: Cardiovascular disease;
CKD: Chronic Kidney Disease;
FCS: Fetal Calf Serum;
Enos: Endothelial Nitric Oxide Synthesis;
Huvecs: Human Umbilical Vein Endothelial Cells;
IS: Indoxyl Sulfate;
Pi: Inorganic Phosphate;
MCP-1: Monocyte Chemoattractant Protein-1;
ROS: Reactive Oxygen Species;
VC: Vascular Calcification;
Vsmcs: Vascular Smooth Muscle Cells

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in patients with chronic kidney disease

(CKD) [1]. Atherosclerosis and vascular calcification (VC) are frequently found in patients with CKD. Furthermore, VC increases the already high risk of cardiovascular mortality in CKD patients. Apoptosis and osteochondrogenictransdifferentiation of vascular smooth muscle cells (VSMCs) have an important role in the development of VC. Endothelial dysfunction is a systemic disorder that can be observed from the early stages of CKD onwards [2]; it is involved in accelerated atherosclerosis and is considered to be an important factor in the elevated CVD risk in CKD [3][4]. The uremic toxins that accumulate in CKD patients as a result of reduced kidney function are harmful compounds associated with endothelial dysfunction and VC [4][5].

Indoxyl sulfate (IS) is a protein-bound uremic toxin that is derived from dietary tryptophan. First, tryptophan is metabolized into indole by tryptophanase within intestinal bacteria (such as Escherichia coli). Following intestinal absorption, the indole is further metabolized to IS in the liver. Since IS is normally excreted via proximal tubular secretion in kidney, the compound accumulates in the blood in CKD patients [6]. Many studies have suggested that IS is involved in the development of CVD. Clinical evidence indicates that high IS levels are associated with an elevated risk of atherosclerosis [7], vascular stiffness, aortic calcification and mortality [8]. Recent research results have provided insight into the underlying mechanism by which IS accumulation contributes to CVD. Muteliefu et al [9] reported that IS stimulates free radical production and induces the osteogenic differentiation of VSMCs. Indoxyl sulfate promotes aortic calcification in hypertensive rats, as evidenced by aortic wall thickening and the expression of osteoblast-specific proteins [10]. Moreover, it has been shown that IS leads to a marked impairment of endothelial cell (EC) function. Dou L et al. found that IS inhibits endothelial proliferation and decreases wound repair [11]. Furthermore, Yang K et al. reported that the release of reactive oxygen species (ROS) and the expression of monocyte chemoattractant protein-1 were enhanced by IS in a dose-dependent manner, whereas cell viability and the production of nitric oxide (NO) were inhibited [12].

Inorganic phosphate (Pi) is also considered to be a uremic toxin because it accumulates when kidney function is impaired. Hyperphosphatemia is a major risk factor for all-cause death, CVD and VC in CKD patients. There is a growing body of evidence to suggest that Pi has a direct effect on VC and endothelial dysfunction. Pi is a well-known inducer of apoptosis and osteochondrogenic differentiation in VSMCs, which ultimately results in VC. Transport of Pi into the cell is primarily mediated by the Pit1 Na/Pi co-transporters and leads to osteochondrogenic differentiation of VSMCs [13]. Increased Pi levels suppress both the expression of growth arrest specific gene 6 and its receptor in VSMCs. Inhibition of this pathway results in the suppression of the phosphatidyl inositol-3 kinase/Akt survival pathway and thus VSMC apoptosis [14]. High levels of Pi are known to induce endothelial dysfunction, since oxidative stress impairs the activity of endothelial nitric oxide synthase (eNOS) [15]. Shuto E et al. showed that increased Pi levels lead to endothelial dysfunction in both animal models and humans [16] via inhibited NO production, increased ROS production and eNOS inactivation in ECs, resulting in impaired endothelium-dependent vasodilation. The researchers also reported that dietary Pi loading caused postprandial elevation of serum Pi and deteriorated endothelium-dependent vasodilation in humans. These findings suggest that elevated IS and Pi levels induce VSMC and EC dysfunction, which in turn contributes to the development of atherosclerosis and VC.

MicroRNAs (miRNAs) are a novel class of small RNAs that downregulate gene expression by binding to corresponding target mRNAs and thus by marking them for translational repression or degradation [17][18]. MiRNAs are strongly expressed in the cardiovascular cells and have important roles in cardiovascular development and biology [19]. Recent research has revealed that dysregulation of miRNAs is involved in vascular system impairments [20]. The miRNA-29 (MiR-29) family has three members (29a, 29b, and 29c). The predicted functions largely overlap, since the MiR-29 family members share a common seed region that primarily determines the target genes [21]. The miR-29 family is involved in the regulation of cell proliferation, differentiation and apoptosis and the expression of several extracellular matrix proteins (including a large number of collagen isoforms, elastin and matrix metalloproteinase (MMP)-2). Dysregulation of the miR-29 family has been reported in atherosclerosis-related diseases, such as myocardial infarction [22], heart failure [23] and stroke [24]. In vitro studies using VSMCs have also shown that miR-29 may be involved in the progression of atherosclerosis [25] and VC [26]. Taken as a whole, these findings suggest that the miR-29 family has an important role in the accelerated progression of atherosclerosis and VC in CKD. However, it is not yet known how miR-29 expression in vessel walls is regulated in CKD.

In the present work, we sought to determine whether or not uremic toxins are associated with changes in miR-29a and miR-29b expression in vascular walls. We first investigated the effects of uremic serum on the expression of miR-29a and miR-29b in HUVECs. We next looked at whether two of the main uremic toxins (Pi and IS) affect this expression. Lastly, we quantified the expression of miR-29a and miR-29b in aortas collected from wild-type mice with or without experimentally induced CKD.

Materials and Methods

Products

The Endothelial Cell Growth Kit - BBE was obtained from

ATCC (Manassas, VA, USA). Indoxyl sulfate, Pi, Dulbecco's Modified Eagle's Medium and penicillin/streptomycin were purchased from Sigma Aldrich (Saint Louis, USA). GlutaMAX™ was obtained from Invitrogen (Saint Aubin, France) and fetal calf serum was from Dominique Dutcher Laboratories (Brumath, France).

Cell Cultures

Human umbilical vein endothelial cells were obtained from ATCC and cultured in a 0.1% gelatin-coated flask with specific culture medium (Vascular Cell Basal Medium) supplemented with the Endothelial Cell Growth Kit - BBE. Cells were maintained at 37°C (5% CO2, 90% humidity) and used for experiments between passage 3 and passage 8.

Sera

Non-uremic and uremic human serum was obtained non-invasively by collecting left-over serum samples taken for biochemical assays from patients (n>30) at Amiens University Medical Center (Amiens, France), and eGFR in all of them was <10ml/min. The sera were dosed for Pi (2.33mM for the normal serum, and 1.75mM for the uremic patients, NS) and indoxylsulfate (3.6μM for the normal serum, and 120.5μM for the uremic patients, $p<0.01$). The fact that Pi levels are not different between normal patients and uremic patients is due to the fact that all patients in Amiens are treated with Phosphate binders. The study's protocol and objectives were approved by the local investigational review board for non-clinical studies(Comité d'Ethique du CHU d'Amiens).

Animals and diets

All animal experiments were performed on female C57 black wildtype (WT) mice (Charles River Laboratories, France). The animals were housed in temperature- and humidity-controlled polycarbonate cages with a 12-hour/12-hour light/dark cycle and were given standard chow diet (Teklad Global Diet 2016, Harlan, UK) and tap water ad libitum. The aorta was dissected down to the renal arteries, removed, frozen into liquid nitrogen and stored until further use. All mice were handled in accordance with French legislation, and the protocol was approved by the local animal care and use committee (CREMEAP).

Animal experiments

At six weeks of age, WT mice were randomly assigned to a CKD group or a non-CKD group. As described elsewhere in detail, a two-step procedure was used to induce CKD [27]. Briefly, we applied cortical electrocautery to the right kidney through a 2-cm flank incision and then performed left total nephrectomy through a similar incision two weeks later. Control animals underwent sham operations, including decapsulation of both kidneys. Special care was taken to avoid damage to the adrenal glands. Mice were sacrificed 10 weeks after the nephrectomy or the sham operation (i.e. at 18 weeks of age). Blood samples were taken before sacrifice and urea levels (10 +/- 1mM for SHAM mice, and 32 +/- 7mM CKD mice, $p<0.001$) and Pi levels (2.2 +/- 0.2mM for SHAM mice, and 3.6 +/- 0.4mM CKD mice, $p<0.05$) were measured. A previous study has shown that, at similar CKD conditions, the levels of serum IS are higher than in control sham mice [28].

RNA isolation and real-time PCR

Experiments were performed as described in [29]. Briefly, RNAs from cells and mouse aorta were isolated with the mirVanaTM Isolation Kit (Applied Biosystems), according to the manufacturer's instructions. TaqMan assays (Applied Biosystems) were used to quantify miRNAs. Quantitative real-time PCRs were then run on a Step One Plus system (Applied Biosystems). The U6 small nuclear RNA was used as an endogenous miRNA control.

Statistical analysis

Results were expressed as the mean ± standard error of the mean. The non-parametric Kruskal-Wallis test was used to probe the statistical significance of intergroup differences in mean values. The Mann-Whitney U test to test the significance of intergroup differences in the sums of ranks. In all tests, the threshold for statistical significance was set to $p<0.05$.

Results

The miRNA expression profile in HUVECs

In order to characterize the expression profile of miRNAs in HUVECs, we selected 10 different miRNAs (including miR-29a and miR-29b) with reference to the known expression profile in vascular ECs and the RNAs' possible involvement in CVD. In HUVECs, six miRNAs (miR-17, miR-29a, miR-29b, miR-92a, miR-221 and miR-222) were seen to be expressed and four (miR-143, miR-145, miR-223 and miR-296) were not (data not shown). Only miR-29a and miR-29b expression was affected by incubation with IS. In view of these results, we chose to focus on the miR-29 family in the rest of the study.

The effect of non-uremic and uremic sera on the expression of miR-29a and miR-29b in ECs

We first examined the effect of incubating HUVECs with 10% non-uremic human serum or 10% uremic human serum on the expression of miR-29a and miR-29b. Incubation with the sera for 24 h (Figure 1A) or five days (Figure 1B) did not affect the expression of miR-29a in

HUVECs (relative to expression in a control experiment with standard culture medium). Although stimulation with sera for 24 h increased miR-29b expression in HUVECs (relative to the control with culture medium), there was no significant difference between cells treated with non-uremic serum and cells treated with uremic serum. Incubation with non-uremic or uremic serum for five days did not affect miR-29b expression (Figure 1).

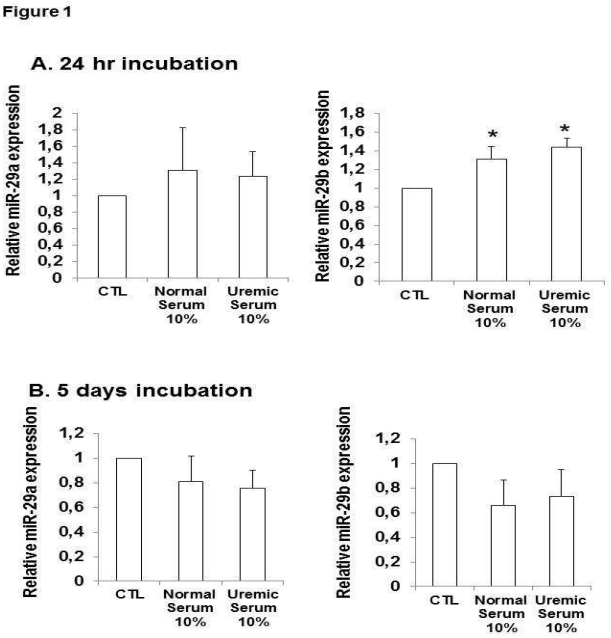

Figure 1. The effects of uremic and non-uremic sera on the expression of miR-29a and miR-29b in vascular cells.
Human umbilical vein endothelial cells were cultured for 24 h(A) or five days (B) with 10% uremic serum or 10% non-uremic serum. Normal medium-treated cells were used as a control. Expression of miR-29a and miR-29b was measured by RT-qPCR. The data shown here correspond to the mean of three independent experiments performed in triplicate. * p<0.05 vs. the control group.

The effect of indoxyl sulfate on expression of miR-29a and miR-29b in HUVECs

We investigated the HUVECs' expression of miR-29a and miR-29b in the presence of various concentration of IS (0, 0.6, 50 or 200μg/ml) for 24 h or five days. According to the report by the European Uraemic Toxin Work Group, 0.6, 50 and 200 μg/ml correspond to the normal concentration of IS in non-uremic patients, the mean uremic concentration in uremic patients and the peak concentration in uremic patients respectively, [28]. In HUVECs, treatment for 24 h significantly decreased the expression of miR-29a (at 50μg/ml IS) and miR-29b (at 0.6, 50 and 200μg/ml IS), relative to a control experiment with standard culture medium (Figure 2A). In contrast, IS treatment for five days did not induce significant changes in miR-29a or miR-29b expression (Figure 2B). We next measured the expression of collagen type1α1, which has been described as a target of the miR-29 family [26]. While mature collagen expression was not affected when cells were incubated with IS for 24 h or five days (not shown), we found a significant increase in levels of its precursor procollagen type 1α1 after five days of IS treatment (Figure 2 C and D).

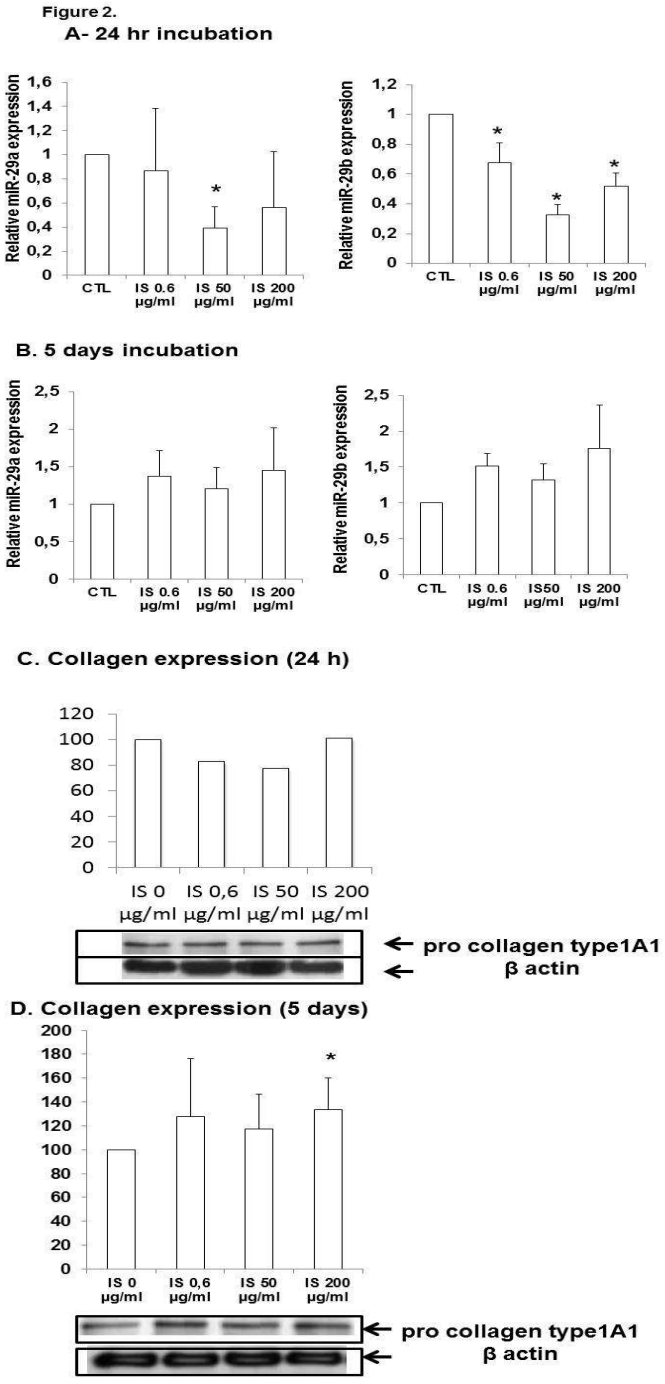

Figure 2. The effect of IS on the expression of miR-29a and miR-29b in vascular cells.
Human umbilical vein endothelial cells were cultured for 24 h (A) or five days (B) with the indicated concentration of IS. Cells treated with normal medium were used as controls. Expression of miR-29a and miR-29b was measured in a RT-qPCR. The data shown here correspond to the mean of three to six independent experiments performed in triplicate. * p<0.05 vs. the control

group. (C) HUVECs were cultured for or five days with the indicated concentrations of IS. Expression of procollagen (an miR29-specific target) was measured using Western blotting and normalized against levels of the housekeeping protein beta actin. * p<0.05 vs. the control group.Two (24 hr time point) to three (5 day time point) independent experiments were performed (a representative experiment is shown here).

The effect of Pi on the expression of miR-29a and miR-29b in HUVECs

We next treated HUVECs for 24 h or five days with 3.0 mM Pi (a concentration reflecting hyperphosphatemia) and measured their expression of miR-29a and miR-29b. A 5 day incubation with Pi (but not a 24 h incubation) significantly increased the expression of both miR-29a and miR-29b, relative to control cells exposed to a physiological concentration of Pi (1.1mM) (Figure 3). Note however that although statistical significance could not be reached, 24 hincubation led also to a marked incresase of both miRNA expression.

Figure 3.

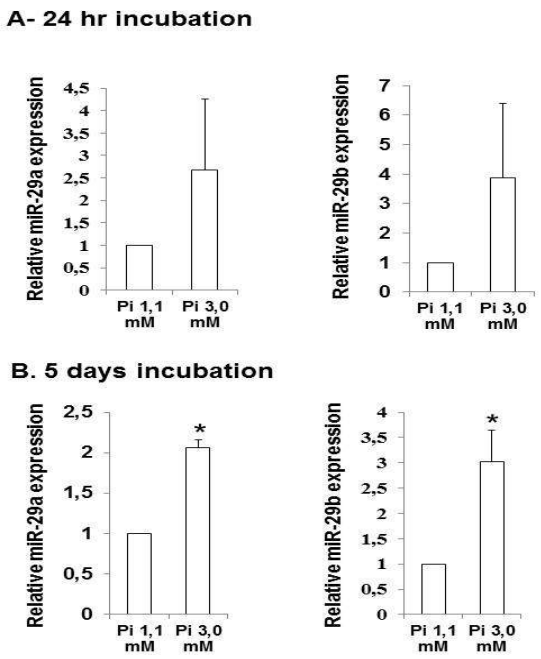

Figure 3. The effect of Pi on the expression of miR-29a and miR-29b in vascular cells.
Human umbilical vein endothelial cells were cultured for 24 h (A) or five days (B) with 3.0 mM Pi. Cells treated with 1.1 mM Pi were used as controls. Expression of miR-29a and miR-29b was measured by RT-qPCR. The data shown here correspond to the mean of three independent experiments performed in triplicate. * p<0.05 vs. the control group.

The effect of CKD on miR-29a and miR-29b expression in mice aorta

We also examined the in vivo fate of miR-29a and miR-29b under uremic conditions. Ten weeks after the experimental induction of CKD or a sham operation in WT mice, we evaluated expression levels of miR-29a and miR-29b in the murine aorta. In line with previous observations by our group [27], the CKD mice exhibited significantly elevated serum urea, calcium and Pi levels (relative to sham-operated mice). These mice have also been associated with a marked development of endothelial dysfunction [30]. The expression of miR-29a and miR-29b was significantly higher in CKD mice than in non-CKD mice (Figure 4).

Figure 4.

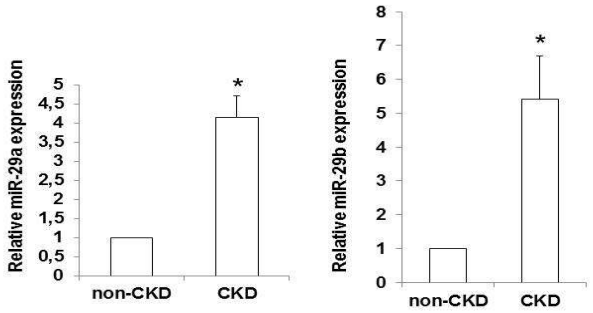

Figure 4. The effect of CKD on the expression of miR-29a and miR-29b in mice.
Ten weeks after the induction of CKD or a sham operation, aortas were removed from CKD and non-CKD mice. The RNAs were extracted and expression of miR-29a and miR-29b was analyzed by RT-qPCR. The data shown here correspond to the mean for three different animals, determined in triplicate. * p<0.05 vs. the control group.

Discussion

In the present study, we selected IS and Pi as representative uremic toxins with involvement in vascular dysfunction. We demonstrated the effects of uremic serum, IS and Pi on miR-29a and miR-29b expression in HUVECs. We found that exposure to IS and Pi is associated with changes in miR-29 expression in this cell type.

Recent evidence suggests that miR-29s have an important role in vessels in general and the progression of atherosclerosis and VC in particular. For example, Chen et al. showed that miR-29b is involved in the progression of atherosclerosis via regulation of MMP-2 and MMP-9 genes [31]. Overactivation of MMPs (which are involved in regulating transmedial elastin degradation in the atherosclerotic media) is regarded as an important contributor to plaque formation and destabilization. Chen et al. also found that oxidized low-density lipoprotein induces upregulation of miR-29b expression, leading to epigenetic modifications of the MMP-2 and MMP-9 genes in VSMCs. Du Y et al. demonstrated that miR-29a and miR-29b downregulate Pi-induced VSMC calcification [26]. Although several reports have highlighted the role of miR-29s in VSMCs, the RNAs' role in vascular ECs is still unknown. Kuen-

bacher A et al. analyzed the expression of 168 miRNAs in HUVECs and found that miR-29s are amongst the miRNAs particularly involved in EC function [32]. Consistently, we detected the expression of miR-29a and miR-29b (together with miR-17, miR-92a, miR-221 and miR-222) in HUVECs. According to the literature, miR-143 and miR-145 are involved in acquisition of the contractile VSMC phenotype [33,34] and miR-223 is associated with VSMC migration and proliferation [29]; as expected, we did not observe the expression of these miRNAs in HUVECs.

When considering uremic toxins, we found that miR-29a and b were upregulated in HUVECs after five days of incubation with 3.0 mM Pi. It is known that high Pi increases apoptosis of ECs [35,36], which in turn results in impaired endothelial integrity. MiR-29s target the genes that down regulate tumor suppressor p53 and apparently serve as apoptosis inducers [21]. MiR-29a and miR-29b were significantly upregulated in HUVECs treated with Pi for five days but for just 24 h. In contrast, we observed downregulation of both miR-29a and miR-29b in HUVECs treated with IS for 24 h, suggesting that (i) miR-29s are involved in several cell functions and (i) an as yet unknown mechanism is involved in regulating miR-29 expression over the timescale of our experiment. We also found that a five-day treatment with IS increased expression of the precursor procollagen type1α1 (a known target for both miR-29a and miR-29b [26]). This is likely to be due to the decrease in miR-29a and miR-29b levels observed after 24 h of incubation with IS. The half-life of collagen is in the order of several days, so a latency of several days for the regulation of miR-29 targets is not unexpected [21].

Another interesting finding of our study is that uremic serum did not induce a significant difference in miR-29 expression in HUVECs (relative to experiments with non-uremic serum). Uremic toxins are defined as waste products that accumulate as a result of impaired kidney function and encompass many different types of compounds. On the basis of a database of 857 publications, the European Uraemic Toxin Work Group listed 90 uremic toxins [37]. Therefore, one can reasonably hypothesize that uremic serum contains not only Pi and IS but a range of uremic toxins that may be variously associated with upregulation or downregulation of miR-29 expression. Hence, the net expression of miR-29s depends on the overall balance. This hypothesis might explain why HUVECs treated with uremic serum and non-uremic serum did not display a significant difference in miR-29a and miR-29b expression, despite the changes produced by incubation with the uremic toxins Pi and IS.

Interestingly, we found that miR-29 expression in mice aorta was upregulated in 10-week-old CKD mice (relative to non-CKD mice). The CKD mice display intense uremia and elevated levels of most uremic toxins (including IS and Pi) [38]. Other miRNAs are known to be deregulated during the course of CKD in humans and rodents [27,31]. Our present study is the first to report that uremia may also be associated with upregulation of miR-29s in the vascular wall. This phenomenon might reflect aging-associated vascular pathologies. Indeed, Boon RA et al. compared aortic tissue expression profiles in elderly mice and young mice; expression of the miR-29 family was upregulated in aged arteries and was negatively correlated with the expression of COL3A1, COL3A1 and ELN [39]. These observations suggest that miR-29 expression in vessels is affected by several factors, including uremic toxins and aging.

There are few literature data on the role of miR-29s in CKD. Wang XH et al. reported that miR-29s are involved in the muscle wasting that accompanies CKD. The researchers observed the downregulation of both miR-29a and miR-29b in muscle from mice with CKD, leading to inhibition of myogenesis [40]. Although the effect of miR-29s on vascular dysfunction in CKD is unclear, medial calcification (a characteristic feature of CKD patients) may be a relevant interesting link. Indeed, medial calcification is associated with degradation of elastin by MMPs. Elastin is a major constituent of the extracellular matrix in the arterial wall, and so degradation increases elastin's affinity for calcium and leads to the growth of hydroxyapatite crystals. The released elastin fragment is also able to bind to elastin receptors on VSMCs and thus directly affect osteogenic differentiation [41]. Since elastin and MMP-2 are downstream targets of miR-29s, the latter may have an important role in the progression of medial calcification.

In conclusion, our present findings suggest that the uremic toxins in uremic serum (including IS and Pi) are variously associated with upregulation or downregulation of miR-29 expression in arterial walls. This finding indicated the complexity of miR-29 regulation in CKD. Upregulation of miR-29s in the CKD mouse aorta suggests that uremia is associated with vascular dysfunction through the regulation of miR-29s in vessel wall cells. Further studies are needed to clarify the regulation of miR-29s in vascular walls and the precise mechanism by which miR-29s are involved in vascular dysfunction in CKD.

Funding:

The present work was funded by grants from the Picardie Regional Council (MARNO-MPCC and MIRNA), including a PhD fellowship for FT and post-doctoral fellowships for HO and EMM.

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