

Physiology and biochemistry of endothelial function in children with chronic renal failure

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Physiology and biochemistry of endothelial function in children with chronic renal failure. Premature atherosclerosis is a major cause of morbidity and mortality in chronic renal failure (CRF). Endothelial dysfunction is a key early event in atherogenesis. The aim of this study was to assess the effect of CRF on endothelial function using physiological and biochemical measures. To focus on the effect of CRF itself, 23 children (matched with 23 controls for age and vessel diameter) were selected because they were normotensive, had normal total cholesterol (TC) levels, and were not on vasoactive drugs. Their mean (range) age was 12.0 (7.8 to 17.0) years; GFR 17.5 (8.8 to 34.5) ml/min/1.73 m². The physiology of endothelial function in the brachial artery was assessed using high resolution ultrasound by measuring its diameter at rest, during reactive hyperemia (endothelium dependent dilation) and after sublingual glyceryl trinitrate (GTN; endothelium independent dilation). Nitric oxide (NO) metabolites and endogenous NO synthetase (eNOS) inhibitors were measured as an assessment of endothelial metabolism. Brachial artery dilation to flow [FMD, mean (SEM)%] was reduced in CRF to 4.9 (0.6) and controls 8.6 (0.6), $P < 0.0001$. In contrast, the response to GTN was similar in both groups: CRF 25.1 (1.6), controls 23.3 (1.2), $P = 0.31$. There was no difference in TC, low density lipoprotein (LDL) or high density lipoprotein (HDL) between the patients and the controls. Triglycerides (TG) were higher in the patients but within the normal range. Antibodies against oxidized LDL (ox-LDL) were high in CRF. Endogenous NOS inhibitors were high in CRF, and intermediate NO metabolites were low. There was no correlation between FMD of the brachial artery and lipid subfractions, or with NO metabolites or eNOS inhibitors. Endothelium dependent dilation of the brachial artery is impaired in children with CRF who do not have co-existing risk factors for atherosclerosis. This may represent early evidence of atherogenic vascular disease.

Premature atherosclerosis is a major cause of morbidity in adults with chronic renal failure (CRF), and is responsible for a mortality rate ten times greater than in the normal population [1]. An increasing number of children with CRF are surviving to adulthood, both because of advances in dialysis and transplantation, and because treatment is being extended to younger patients. Their long-term morbidity and mortality are uncertain, but they

might be expected to have similar vascular complications to adults with CRF, at an even earlier age.

Although the clinical manifestations of atherosclerosis do not usually occur before adulthood, the process begins in childhood [2]. Endothelial dysfunction is a key early event that precedes the formation of atherosclerotic plaques, and results in reduced bioavailability of nitric oxide (NO), which may be an important anti-atherogenic agent [3]. In CRF, abnormal endothelial function and NO activity may result from both the metabolic consequences of CRF, such as reduced clearance of endogenous NO synthetase (eNOS) inhibitors [4, 5] and increased oxidative stress [6], as well as from the presence of other classical risk factors such as hyperlipidemia and hypertension [1].

We have developed a non-invasive technique using high resolution ultrasound to assess vascular reactivity in the conduit arteries of the systemic circulation, which can be used to study endothelial function from as early as the first decade of life [7]. We have previously shown that endothelial dysfunction may occur before clinical evidence of vascular disease in subjects with hypercholesterolemia [8], diabetes [9] and in cigarette smokers [10].

In the current study we have examined the influence of CRF on endothelial function in young subjects. In order to avoid confounding variables, we purposefully selected subjects with CRF who were not smokers, hypertensive, hypercholesterolemic or diabetic, and were not receiving vasoactive drug therapy.

Our findings suggest that CRF has a direct adverse effect on endothelial function in this young patient group. This may influence later morbidity and mortality from large vessel atherosclerotic disease independently of other risk factors.

METHODS

Patients

Twenty-three children (18 boys), aged 7.8 to 17.0 years (median and mean 12.0), with CRF on conservative management, none of whom were on maintenance dialysis [mean (range) glomerular filtration rate (GFR) 17.5 (8.8 to 34.5) ml/min/1.73 m²], were studied. Their diagnoses were renal dysplasia (19), reflux nephropathy (2), Alport's syndrome (1) and focal glomerulosclerosis (1). They were selected from an outpatient population because they were normotensive [mean (SEM) systolic blood pressure SD score (BPSDS) for age -0.04 (0.15), diastolic -0.11 (0.18)]; with

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plasma total cholesterol (TC) < 5.2 mmol/liter and low density lipoprotein (LDL) < 3.3 mmol/liter; were not diabetic or nephrotic [mean (SEM) serum albumin 42.9 (0.6) g/liter, 24-hr protein excretion 0.85 (0.2) g/liter]; and were not taking vasoactive or lipid-lowering medications. They were matched with 23 control subjects (friends or relatives of hospital staff) for age and brachial artery diameter. Twenty of the controls were gender matched with the CRF patients (87%). Ten boys and 3 girls in the CRF group, compared to 9 boys and 4 girls in the controls were over the normal age for onset of puberty (over 11 years in boys and 10 years in girls).

Plasma nitrite and nitrate (NO oxidation products), high and low molecular weight nitrosothiols (intermediate metabolites of NO), eNOS inhibitors [asymmetrical dimethylarginine (ADMA) and symmetrical dimethylarginine (SDMA)] in the CRF patients were compared to values from 6 control children, aged 6 to 16 (median 11 years), who were having blood taken for family genetic screening of non-cardiac abnormalities. Plasma TC, triglycerides (TG), LDL and high density lipoprotein (HDL) were measured in the CRF group and in 12 of the controls who were willing to have blood tests. Other lipid subfractions such as Apo A1, ApoB, lipoprotein(a) [Lp(a)] and antibodies against oxidized LDL (ox-LDL) were measured in the CRF group. All blood samples were taken after an overnight fast (although tap water was allowed), were immediately centrifuged, and the plasma was stored at -70°C. Each subject and/or their parents gave informed consent to the study, which was approved by the Local Committee on Ethical Practice.

Endothelial function study

Endothelial and smooth muscle function were studied non-invasively by examining brachial artery responses to endothelial dependent and independent stimuli as we have previously reported [7]. Serial diameter changes were measured at rest, in response to reactive hyperemia (with increased flow producing endothelial dependent vasodilatation), again at rest, and finally after sublingual glyceryl trinitrate (GTN), which is an endothelial independent vasodilator. All subjects were scanned in a supine position following a 10-minute rest period. A high resolution B-mode ultrasound image of the brachial artery was obtained in a longitudinal section, 5 to 10 cm above the antecubital fossa, using a 7 MHz linear array transducer and Acuson 128XP/10 system (Acuson, Mountain View, CA, USA), connected to a wall-tracking system (Ingenious Systems, Netherlands) allowing accurate on line diameter measurements. The center of the artery was identified when the clearest picture of the anterior and posterior vessel wall layers was obtained. Depth and gain settings were set to optimize the lumen/arterial wall interface, and machine operating parameters were not changed throughout the study. The arm remained in the same position and a satisfactory transducer position was maintained using a stereotactic clamp. All the ultrasound scan data were recorded on superVHS video for later flow analysis and all scans were performed by the same operator. To measure the brachial artery diameter an M-line was placed perpendicular to the vessel walls on the B-mode image, the radio-frequency signals from the M-mode output were then relayed to the wall-tracking system. On completion of five seconds of data acquisition, the first radio-frequency signal was displayed and electronic markers placed at the vessel wall/lumen interface and a beat by beat computation of the end diastolic diameter and

mean over 5 to 10 cardiac cycles was obtained. Reproducibility and repeatability of this method have been previously reported [11, 12]. A resting scan was recorded and arterial flow velocity was measured using a pulsed Doppler signal at a 70° angle to the vessel with the range gate (1.5 mm) in the center of the artery. Volume blood flow was calculated by multiplying the velocity time integral of the Doppler flow signal (corrected for angle) by the heart rate and the vessel cross-sectional area (πr^2). A pneumatic tourniquet placed around the forearm was then inflated to 300 mm Hg for 4.5 minutes followed by rapid release, inducing increased flow. The post-hyperemic diameter was measured between 55 and 65 seconds after cuff deflation. Peak reactive hyperemia was calculated as the maximal flow change within 15 seconds of cuff deflation divided by the flow during the resting (baseline) scan reported as percentage increase in flow. A further reactive hyperemia was calculated in the same way 15 seconds after cuff release. Since the velocity is taken from the center of the artery, absolute values may be overestimated, but the relative values before and after cuff inflation are accurate. A further 10 minutes was allowed for vessel recovery, after which a second resting scan was recorded. A 400 µg sublingual dose of GTN was then administered and a final scan was recorded three minutes later. Flow mediated dilation (FMD) in the brachial artery following reactive hyperemia and endothelium independent dilation following GTN administration were expressed as percentage diameter change relative to the first base line scan.

To assess the reproducibility of the ultrasound technique in CRF, seven of the children were seen on three occasions, all within four months of the first study.

Lipid analysis

TC was measured using the cholesterol C system high performance cholesterol oxidase 4-aminophenazone (CHOD-PAP) method and TG by glyceryl phosphate oxidase 4-aminophenazone (GPO-PAP) high performance enzymatic colorimetric test (both Boehringer Mannheim Diagnostica GmbH, Mannheim, Germany) [13]. HDL was measured following precipitation of ApoB containing lipoproteins and LDL was calculated using the Friedewald formula [14]. ApoA1 and ApoB were measured using immunoturbidimetry (Immuno Ltd, Sevenoaks, Kent, UK) [15], and Lp(a) by enzyme-linked immunosorbent assay (ELISA) (Immuno Ltd) [16]. All assays were validated by the National External Quality Assessment Scheme. Antibodies against ox-LDL were measured by ELISA with a 450 nm filter, based on a set of standardized serum and controls obtained from a O-lab-ELISA kit (Biomedica Gruppe, Austria) [17].

Nitric oxide biochemistry

Nitrite and nitrate were measured using high performance capillary electrophoresis [18]. Nitrosothiols were measured after separating the plasma into two molecular weight fractions by ultracentrifugation (5,000 Mwt filter at 5000 g). Mercury salts were then added to displace the NO from the thiol to generate nitrite, which was assayed by capillary electrophoresis [18].

ADMA and SDMA were measured by high performance liquid chromatography using electrochemical detection after precolumn derivatization with o-phthalaldehyde (OPA)/b-mercaptoethanol [19].

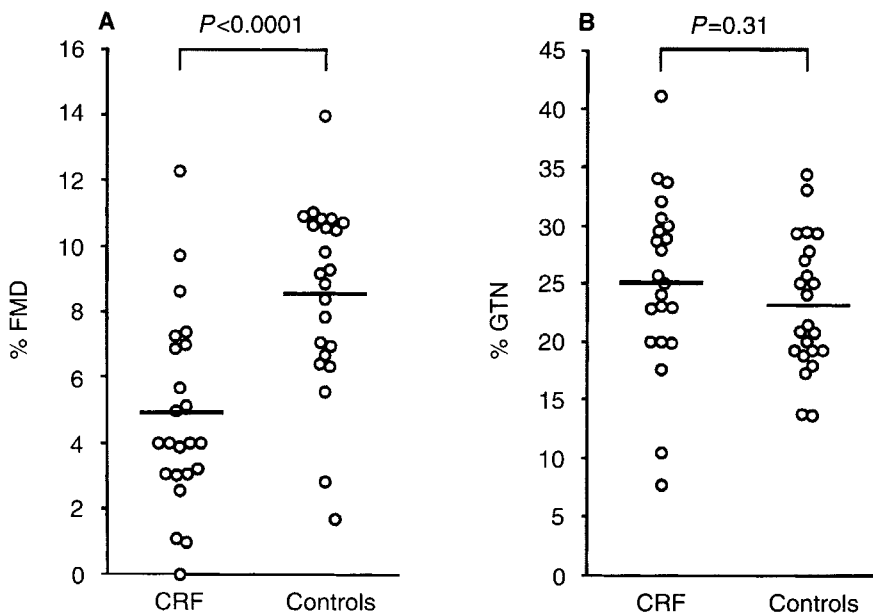


Fig. 1. Flow mediated (FMD, *A*) and glyceryl trinitrate (GTN, *B*)-induced dilation in the controls and children with CRF. Horizontal lines are group means. FMD was significantly impaired in CRF whereas GTN response was normal.

Table 1. Vascular study results in children with CRF and controls

	CRF	Controls	<i>P</i>
Peak reactive hyperemia %	284.4 (23.7)	357.6 (29.3)	0.06
Reactive hyperemia at 15 seconds %	262.3 (27.2)	241 (24.5)	0.61
Vessel size <i>mm</i>	2.9 (0.1)	2.9 (0.1)	0.72
FMD %	4.9 (0.6)	8.6 (0.6)	<0.0001
GTN %	25.1 (1.6)	23.3 (1.2)	0.31

Results are expressed as mean (SEM).

Table 2. Nitric oxide biochemistry results

	CRF	Controls	<i>P</i>
Nitrate μM	100.9 (9.4)	32.1 (4.3)	<0.00001
Nitrite μM	1.2 (0.1)	1.4 (0.2)	0.38
ADMA μM	3.8 (0.4)	0.7 (0.1)	0.001
SDMA μM	1.8 (0.2)	0.3 (0.1)	0.001
Low molecular wt nitrosothiol μM	0.9 (0.1)	1.6 (0.2)	0.02
High molecular wt nitrosothiol μM	2.5 (0.1)	3.4 (0.3)	0.0001

Results are expressed as mean (SEM).

Statistics

Descriptive statistics are expressed as mean \pm SEM. The CRF and control groups were compared using two sample *t*-tests. Univariate regression analysis was used to assess the relationship between the two dependent variables, flow mediated and GTN induced dilation, and sex, age, vessel size, and TC in all the children, and with TG, HDL, LDL, ApoA1, ApoB, Lp(a), antibodies against ox-LDL, GFR and NO metabolites in the 23 children with CRF. Variability between results of the high resolution ultrasound technique (repeatability) for the seven patients who had been studied on more than one occasion was calculated as ratio of the within subject SD (the square root of the residual mean square taken from analysis of variance for repeated measures) to the overall mean. This gives the estimated coefficient of variation (%). Statistical significance was inferred at a *P* value of < 0.05.

RESULTS

Endothelial physiology

There was no difference in resting vessel size, peak reactive hyperemia, or reactive hyperemia at 15 seconds after cuff release between CRF and controls (Table 1). However, FMD(%) in CRF was significantly impaired in comparison to the controls [4.9 (0.6) vs. 8.6 (0.6), *P* < 0.0001]. In contrast, dilation to GTN did not differ from the controls [25.1 (1.6) vs. 23.3 (1.2), *P* = 0.31] (Fig. 1).

On univariate analysis, there was no correlation between endothelium dependent (FMD) or independent brachial artery dilation and measures of renal function, lipid subfractions and NO biochemistry.

Study of repeatability

For each child, there was a directionally similar response to increased flow and to GTN with reproducible failure to dilate to increased flow on the three study occasions. The mean (range) across visits of observed FMD was 5.1% (1.5 to 7.3). From analysis of variance for repeated measures, the estimated coefficient of variance between visits was 4.3%.

Nitric oxide biochemistry

Plasma nitrate, ADMA and SDMA levels were significantly elevated in the CRF children (Table 2). There was a significant inverse correlation between GFR and total dimethylarginines ($r = -0.48$, *P* = 0.03) and SDMA ($r = -0.46$, *P* = 0.04), although the correlation with ADMA was not significant ($r = 0.41$, *P* = 0.07). Nitrite levels however did not differ from controls. Low and high molecular weight nitrosothiol levels were significantly lower in the CRF group, and there was a positive correlation between GFR and low molecular weight nitrosothiol ($r = 0.52$, *P* = 0.017).

Although it would be expected that high levels of eNOS inhibitors might decrease NO production, there was no correlation between ADMA and SDMA and any NO metabolites.

Table 3. Lipid subfractions

	CRF	Controls	Normal values	P
Age years	12.1 (0.5)	12.6 (0.5)		0.53
Total cholesterol mmol/liter	5 (0.1)	4.8 (0.3)	<5.2	0.52
Low density lipoprotein mmol/liter	3.0 (0.1)	3.0 (0.2)	<3.3	0.97
High density lipoprotein mmol/liter	1.2 (0.04)	1.2 (0.04)	1.0–2.0	0.92
Triglycerides mmol/liter	1.5 (1.1)	0.76 (1.2)	<1.7	0.003
Antibodies against OX-LDL μ /ml	260 (37.5)		<250	
ApoA1 g/liter	1.6 (0.03)		0.7–1.7	
ApoB g/liter	1.0 (0.04)		0.6–1.4	
Lp(a) g/liter	0.12 (0.07)		<0.3	

Results are expressed as mean (SEM).

Lipid studies

There was no significant difference between TC, HDL and LDL levels in the patients and the controls (Table 3). However, TG levels were higher in the CRF patients and antibodies against ox-LDL were elevated. GFR correlated with TC ($r = 0.50$, $P = 0.026$) and log TG ($r = -0.64$, $P = 0.001$) levels even when age was taken into consideration, but not with other lipid subfractions.

DISCUSSION

Our results show that impaired endothelial function is already present in the conduit arteries of children with CRF by the first decade of life. It is likely that this represents an early manifestation of the atherosclerotic process, which causes important morbidity and mortality in CRF patients in later life [1].

A number of factors may contribute to endothelial dysfunction in CRF, including dyslipidemia [1], drug therapy, increased oxidative stress [6] and the metabolic consequences of CRF themselves. In this study, we set out to determine the influence of CRF on endothelial function as directly as possible by excluding patients with hypertension, high plasma cholesterol levels and those receiving vasoactive drugs. We were able to study endothelial function from a very early stage before acquired risk factors are likely to play a major role, because of the availability of a non-invasive technique to examine vascular physiology in conduit arteries of the systemic circulation. Vasodilation to increased flow (an endothelium dependent stimulus) is contrasted with response to GTN (which acts independently of the endothelium). This technique, both in earlier studies and in the CRF patients, has been shown to be accurate and reproducible [11, 12]. As FMD in the brachial artery can be attenuated by intra-arterial infusion of L-NMMA, it is likely that this method assesses the integrity of the L-arginine/NO pathway in conduit arteries [20]. Furthermore, a close correlation has been demonstrated between endothelial function in the brachial artery, assessed using our method, and endothelial function in the coronary arteries assessed invasively using acetylcholine [21].

NO not only acts as a physiological regulator of vascular tone [22], but it is also an important anti-atherogenic molecule that inhibits platelet activation, monocyte and endothelial cell interaction, and smooth muscle cell proliferation [3]. We chose to use a physiological measure of NO-dependent endothelial function because biochemical measurements are difficult to interpret in

CRF due to the effects of abnormal renal clearance. This may explain the lack of correlation between measures such as nitrite and nitrate and FMD. Interestingly, nitrosothiols were not affected by renal clearance, and were lowest in the patients with the lower GFR. S-nitrosothiols, such as S-nitrosocysteine and S-nitrosoglutathione, are formed either by S-nitrosation of free thiol groups in the presence of NO [23] or by the reaction of thiols with peroxynitrite, which is derived from the reaction of NO with superoxide anion [24]. The nitrosothiols have been shown to have biological properties similar to those of NO [25], which may be released from them [26]. While the biological significance of S-nitrosothiols remains unclear, they may represent a measure of NO bioavailability, and low levels in CRF may be one mechanism whereby NO activity is impaired.

In this study, we did not measure other endothelial dependent vasoactive compounds, such as endothelin-1 and thromboxane-A₂, because neither has been shown to have a major role in clinical vascular disease [27]. Our findings of markedly reduced FMD in young subjects from as early as the first and second decades of life indicate that CRF may be contributing to endothelial abnormalities in addition to the influences of other vascular risk factors.

Endothelial dysfunction in CRF may involve abnormalities of both NO production and breakdown. Decreased synthesis may be due to the presence of elevated levels of L-arginine analogues such as ADMA and SDMA in CRF in proportion to its severity, which competitively antagonize eNOS, accumulate in CRF and correlate with its progression [4, 5]. They have been shown experimentally to increase vascular tone [5] and promote early atherogenic changes [28]. Other molecules that accumulate in uremia, such as the cytokine IL-8, also inhibit eNOS [4]. Additionally, in CRF circulating levels of L-arginine, the substrate for NO production, are reduced [5]. Increased inactivation of NO may also be important with increased oxidative stress and free radical production in CRF [6]. While total LDL in our patients did not differ from the controls (as we had excluded children with hypercholesteremia), levels of antibodies to ox-LDL were elevated in our patients with CRF. Ox-LDL is a critical factor in promoting atherogenesis [17] because it interferes with NO metabolism [29], promotes monocyte chemotaxis and transformation, and has a direct effect on endothelial cell survival [30]. Thus, in CRF as in other high risk factor groups, such as insulin-dependent diabetes mellitus, LDL levels even within the normal range may have an impact on endothelial function [9]. A similar situation thus may apply in the children with CRF. In addition, the higher TG levels in the CRF patients may play a role in endothelial dysfunction, but their influence on atherogenesis remains controversial [1].

In conclusion, even young children with CRF, whose outlook for vascular disease would be expected to be relatively good because of the absence of hypertension and hypercholesteremia, have evidence of endothelial dysfunction that may be an early manifestation of atherogenesis. Detection at this early stage of abnormal vascular function permits serial studies of interventions such as risk factor modification, anti-oxidants [31], or L-arginine administration [8], aiming to prevent or retard large vessel atherosclerosis, which is such an important contributor to clinical morbidity and mortality in these patients.

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