Nitric Oxide Production Is Reduced in Patients With Chronic Renal Failure

Robert Wever, Peter Boer, Michel Hijmering, Erik Stroes, Marianne Verhaar, John Kastelein, Kees Versluis, Fija Lagerwerf, Herman van Rijn, Hein Koomans, Ton Rabelink

Abstract—In patients with chronic renal failure (CRF), atherosclerosis is a major cause of cardiovascular morbidity and mortality. Generally, atherosclerosis has been associated with a reduced bioavailability of nitric oxide (NO). Experimental studies have indicated the presence of enhanced NO degradation by reactive oxygen species as well as decreased NO production as possible causes for this reduced NO bioavailability. So far, the question whether or not NO production is impaired in patients with CRF has never been investigated. Therefore, we measured whole body NO production in 7 patients with CRF, and in 7 matched healthy subjects. To assess the relative importance of a dysfunction of NO synthase (NOS), we compared the NO production of these patients to that of 2 other groups known to have endothelial dysfunction, ie, 7 patients with familial hypercholesterolemia (FH) who did not yet have signs of clinical cardiovascular disease (all nonsmokers), and 5 cigarette smokers. These groups were also compared with 7 nonsmoking, age-matched healthy subjects. Whole body NO production, determined as in vivo arginine-to-citrulline conversion, was assessed by giving an intravenous infusion of \[^{15}\text{N}_2\]-arginine as a substrate for NOS and measuring isotopic plasma enrichment of \[^{15}\text{N}\text{-citrulline by LC-MS. NO production in the CRF patients (0.13±0.02 \text{ mmol kg}^{-1} \text{ h}^{-1}) was significantly lower (P<0.05) than in the corresponding control group (0.23±0.09 \text{ mmol kg}^{-1} \text{ h}^{-1}). NO production also tended to be lower in the FH patients (0.16±0.04 \text{ mmol kg}^{-1} \text{ h}^{-1}), but the difference with the corresponding control group did not reach significance (0.22±0.06 \text{ mmol kg}^{-1} \text{ h}^{-1}). In the group of smokers, NO production was similar to that in nonsmokers (0.22±0.09 \text{ mmol kg}^{-1} \text{ h}^{-1}). In conclusion, it is demonstrated for the first time that basal whole body NO production is reduced in patients with CRF. This finding implies that therapeutic interventions to endothelial dysfunction in these patients should be primarily directed toward improvement of NO production. The finding of only a tendency toward reduction of NO production in patients with FH and the absence of a reduction in cigarette smokers suggests that other mechanisms such as enhanced NO degradation may be involved in the decrease of NO bioavailability in these groups. (Arterioscler Thromb Vasc Biol. 1999;19:1168-1172.)

Key Words: nitric oxide ■ chronic renal failure ■ atherosclerosis ■ endothelium ■ hypercholesterolemia

Premature atherosclerosis is one of the primary causes of morbidity and mortality in patients with chronic renal insufficiency.\(^1\) Over the last decade endothelial dysfunction has been identified as an early mediator in this process. Nitric oxide (NO) is one of the main factors involved in the antiatherosclerotic effects of the endothelium;\(^2\) and chronic renal failure (CRF) has been associated with impaired NO bioavailability in the absence of concomitant risk factors.\(^3\)-\(^5\) However, the finding of a reduced NO bioavailability as demonstrated by functional studies does not provide insight into the mechanisms causing endothelial dysfunction, because reduced bioavailability can be the result of decreased NO production, increased NO degradation, or both. In patients with CRF, NO production can be reduced by a diminished NO synthase (NOS) activity, which in turn can be the result of a decreased clearance of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA);\(^7\)-\(^9\) or a decreased bioavailability of the NOS substrate \(L\)-arginine.\(^10\)-\(^12\) On the other hand, CRF has also been associated with enhanced concentrations of oxygen radical species that can inactivate NO.\(^13\)-\(^15\) NOS incorporates molecular oxygen into the guanidino group of \(L\)-arginine, yielding NO and \(L\)-citrulline.\(^16\) By utilizing this reaction, NO production by NOS can be monitored by measuring the isotopic enrichment of \[^{15}\text{N}\text{-citrulline in plasma during intravenous infusion of \[^{15}\text{N}\text{-arginine.\(^17\) This reaction is specific for NOS and discriminates from alternative \(L\)-citrulline formation via the urea cycle pathway.\(^17\) Unlike NOS-derived citrulline, the \[^{15}\text{N}\text{-label is not retained in urea cycle-derived citrulline because the complete

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guanidino group of arginine is removed yielding ornithine, which then condenses with carbamoyl phosphate to give citrulline. Unlike measurements with an NO electrode, which measures net NO release, this method gives direct information on the enzymatic activity of NOS in vivo. To investigate NO production in patients with CRF, we used a recently developed LC-MS technique to quantitate whole body NO production. We studied a matched group of healthy subjects as controls. In addition, to judge the severity of a possible dysfunction of NOS in patients with CRF, we determined NO production in 2 other groups known to have impaired NO bioavailability, ie, patients with familial hypercholesterolemia (FH), at a stage at which clinical cardiovascular disease was not yet present,3–5 and in cigarette smokers.18,19 The latter groups were also compared with a corresponding age-matched control group.

### Methods

#### Subjects

In total, in vivo NO production was assessed in 33 subjects (Table 1). Seven patients with CRF participated in the study. Causes of renal failure were nephroclerosis (3), chronic interstitial nephritis (2), and polycystic kidney disease (2). None of the patients were on hemodialysis; 3 were cigarette smokers; 4 had manifest cardiovascular disease, as indicated by angina pectoris (2), claudicatio intermittens (2), and abdominal aortic aneurism (1). Four patients received concomitant antihypertensive therapy, ie, angiotensin converting enzyme inhibition (3), calcium channel entry blockers (2), and diuretics (3). The CRF patients were compared with a control group of 7 age-matched healthy subjects (Control 1). The group of FH patients also consisted of 7 subjects, all of them nonsmokers. None of them had signs of cardiovascular disease on clinical examination and ECG. Any medication had been discontinued for a period of 2 weeks. They were compared with an age-matched control group of 7 healthy subjects, also nonsmokers (Control 2). The FH patients and the corresponding control group were also compared with a group of 5 cigarette smokers. All smokers had refrained from smoking for at least 24 hours. The study protocol was approved by the institutional committee on ethics for studies in humans. The subjects had given written informed consent after the aim of the study had been explained to them.

#### Study Protocol

The subjects were fasting at the time of study. After blood sampling for determination of the background isotope ratios of plasma arginine and citrulline, a priming dose of 12 μmol l−1-[guanidino-15N2]-arginine (purity >98%; Mass Trace) per kg body weight was given, followed by a constant infusion for 120 minutes of 11.2 μmol·h−1·kg−1 body weight. Under these conditions, plasma enrichments of [15N2]-arginine and [15N]-citrulline reached steady state levels in 30 to 60 minutes. Blood samples for measurement of [15N2]-arginine and [15N]-citrulline enrichments were collected every 30 minutes and immediately centrifuged at 4°C. Plasma samples were stored at −20°C.

#### HPLC-MS

Arginine-to-citrulline conversion rates were measured by HPLC-MS. Briefly, plasma samples were deproteinized, chromatographed on Dowex AG-50W-X4 cation exchange columns, and eluted with 4 mol/L ammonia. The eluates were dried under nitrogen, derivatized to benzylesters by heating with benzyl alcohol: acetylchloride 4:1 (vol/vol) at 45°C for 2 hours, extracted with 1 mmol/L acetic acid, dried, and redissolved in 0.06% trifluoroacetic acid. HPLC separations were performed on a Pharmacia Smart System with a 4×250 mm Sephasil C18 column. Peaks were monitored with UV-detection at 214 nm. Aliquots were injected and eluted isocratically with acetonitrile: water: trifluoroacetic acid 15:85:0.06 (vol/vol/vol) at a flow rate of 0.4 mL/min. The benzyllarginine and benzylcitrulline fractions were collected, dried, and redissolved. A VG Platform single quadrupole mass spectrometer was used for positive ion electro-spray ionization mass spectrometry. Aliquots of the redissolved fractions were injected at a solvent flow of 30 μL/min, and the m/z range from 260 to 270 was scanned. The isotope ratios m/z 267/266 and m/z 267/265 were calculated for arginine and citrulline, respectively. The detection limit of plasma citrulline enrichment was 0.09 atom percent excess (APE). Analysis of plasma samples spiked with [15N]-citrulline (range 0.12 to 2.40 APE) showed good agreement between observed and calculated enrichments (Y=1.049X+0.045, r=0.9985).

#### Calculations

Plasma isotope enrichments were expressed as:

\[ \text{APE} = \frac{100 \times (r_{m} - r_{n})}{(r_{m} - r_{n} + 1)} \]

where \( r_{m} \) is the isotope ratio of the enriched sample, and \( r_{n} \) is the background ratio of the preinfusion sample. Plasma arginine fluxes were calculated from plasma arginine enrichment during steady state infusion, using the single pool model for flux:

\[ Q_{arg} = I_{arg} \times \left( \frac{\text{APE}_{inf} - \text{APE}_{arg}}{\text{APE}_{inf}} \right) - 1 \]

where \( Q_{arg} \) is the arginine flux (μmol·kg−1·h−1), \( I_{arg} \) is the infusion rate of [15N2]-arginine (μmol·kg−1·h−1), and \( \text{APE}_{inf} \) is the enrichment of

### Table 1. Characteristics of Patients and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>CRF Patients</th>
<th>Control 1</th>
<th>FH Patients</th>
<th>Control 2</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Male/female</td>
<td>6/1</td>
<td>5/2</td>
<td>7/0</td>
<td>7/0</td>
<td>5/0</td>
</tr>
<tr>
<td>Nonsmokers/smokers</td>
<td>4/3</td>
<td>5/2</td>
<td>7/0</td>
<td>7/0</td>
<td>0/5</td>
</tr>
<tr>
<td>Age, y</td>
<td>62±18*</td>
<td>62±20*</td>
<td>36±12</td>
<td>29±6</td>
<td>25±3</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>77±12</td>
<td>77±10</td>
<td>77±8</td>
<td>79±11</td>
<td>80±10</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>142±27</td>
<td>130±9</td>
<td>126±10</td>
<td>121±6</td>
<td>124±8</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>79±14</td>
<td>77±8</td>
<td>73±8</td>
<td>70±5</td>
<td>72±6</td>
</tr>
<tr>
<td>Plasma creatinine, μmol/L</td>
<td>770±250*</td>
<td>82±9</td>
<td>77±11</td>
<td>79±10</td>
<td>81±10</td>
</tr>
<tr>
<td>Plasma arginine, μmol/L</td>
<td>71±21</td>
<td>95±26</td>
<td>85±15</td>
<td>94±19</td>
<td>92±11</td>
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<tr>
<td>Plasma triglycerides, mmol/L</td>
<td>2.1±1.0</td>
<td>1.7±0.9</td>
<td>2.3±2.0</td>
<td>1.4±0.7</td>
<td>0.9±0.1</td>
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<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.6±0.4</td>
<td>5.3±1.4</td>
<td>8.7±2.0*</td>
<td>4.4±0.9</td>
<td>4.1±1.1</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.0±0.3</td>
<td>1.0±0.2</td>
<td>0.9±0.2</td>
<td>1.1±0.3</td>
<td>1.2±0.3</td>
</tr>
</tbody>
</table>

CRF indicates chronic renal failure; Control 1, control group for CRF patients; FH, familial hypercholesterolemia; Control 2, control group for FH patients; Smokers, control group of cigarette smokers.

*P<0.01 with respect to other groups.
TABLE 2. Steady State Plasma Enrichments of \([{}^{15}\text{N}_2\text{-Arginine} and \([{}^{15}\text{N}]\text{-Citrulline During Infusion of \([{}^{15}\text{N}_2\text{-Arginine, Calculated Arginine Flux, and NOS Activity Determined as Arginine-to-Citrulline Conversion Rate}\]

<table>
<thead>
<tr>
<th>CRF Patients</th>
<th>Control 1</th>
<th>FH Patients</th>
<th>Control 2</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma arginine enrichment (APE)</td>
<td>13.6±4.1</td>
<td>11.6±0.4</td>
<td>12.2±2.0</td>
<td>11.9±1.9</td>
</tr>
<tr>
<td>Plasma citrulline enrichment (APE)</td>
<td>0.21±0.10</td>
<td>0.31±0.13</td>
<td>0.22±0.04</td>
<td>0.30±0.10</td>
</tr>
<tr>
<td>Plasma arginine flux ((\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}))</td>
<td>76±26</td>
<td>86±4</td>
<td>80±13</td>
<td>85±15</td>
</tr>
<tr>
<td>NOS activity ((\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}))</td>
<td>0.13±0.02*</td>
<td>0.23±0.09</td>
<td>0.16±0.04</td>
<td>0.22±0.06</td>
</tr>
</tbody>
</table>

*APE indicates atom percent excess; other abbreviations as in Table 1.

Statistics
Data are presented as mean±SD. Differences between groups were evaluated by Kruskal-Wallis 1-way analysis of variance on ranks and Dunn’s test for multiple comparisons. A value of \(P<0.05\) was considered significant.

Results
Basal preinfusion plasma arginine levels are given in Table 1. There were no significant differences between the 5 groups, albeit that the CRF group tended to be lower than the other groups. The results of the \([{}^{15}\text{N}_2\text{-Arginine infusion studies are shown in Table 2. Steady state plasma \([{}^{15}\text{N}_2\text{-arginine enrichments as well as arginine plasma fluxes were similar in the 5 groups. Steady state plasma \([{}^{15}\text{N}]\text{-citrulline enrichments were lower in the CRF and FH groups as compared with the control groups and smokers, but this was not statistically significant. Mean NOS activities (measured as arginine-to-citrulline conversion rates) are also shown in Table 2. In the patients with CRF, mean NOS activity was significantly lower (\(P<0.05\)) than in the corresponding control group. In the patients with FH, mean NOS activity tended to be lower than in the corresponding control group, but this difference did not reach statistical significance. There were no significant differences between control groups 1 and 2, nor between smokers and nonsmokers. In the Figure, the NOS activities of all groups are presented in a Box-Whisker plot.

Discussion
Using a novel method to measure NO production, we demonstrate for the first time that whole body NO production is reduced in patients with CRF. Endothelial dysfunction is an early event in the development of atherosclerosis in CRF, and is known to precede formation of atherosclerotic plaques. Our data indicate that the resulting reduction in NO bioavailability in CRF is caused by decreased NO production. In our patients with FH, NO production only showed a tendency to decrease, indicating that increased NO degradation is at least partially responsible for the decreased NO bioavailability that has been reported in hypercholesterolemia. In cigarette smokers NO production was normal, which suggests that increased NO degradation is the major determinant of the decreased NO bioavailability that has been reported in this group. Notably, some of the CRF patients had manifest atherosclerosis, which is almost invariably present in adults with CRF. Atherosclerosis itself may be one of the causes of the impaired NO production, because a reduced expression of e-NOS enzyme has recently been reported to occur in conditions of atherosclerosis. Alternatively, impaired NO production may also have resulted from accumulation of the endogenous NOS-inhibitor ADMA in patients with end-stage renal disease, which is well documented. Furthermore, the bioavailability of the NOS substrate L-arginine has been found to be decreased in subtotally nephrectomized rats as well as in dialysis patients, possibly as a result of malnutrition or arginine loss caused by hemodialysis. In the present study, plasma L-arginine levels of the CRF patients also tended to be lower than that of the other groups, although the differences were not significant. Conceivably, decreased L-arginine levels may become a rate-limiting factor for NOS activity. However, reduced NO bioavailability in patients with CRF.

Contrary to our findings in patients with CRF, we found NO production to be less disturbed in patients with FH.
reduced basal as well as receptor-dependent NO bioavailability has been consistently reported in patients with FH by many groups, including our own.3–5 NO bioavailability may be decreased secondary to G protein uncoupling36 or reduced NOS expression by oxidized LDL.31 In addition, increased ADMA levels32,33 as well as reduced bioavailability of L-arginine have been found in hypercholesterolemia.41–43 Our data support the notion that in the early phase of hypercholesterolemia, decreased NO bioavailability is probably indeed a multifactorial phenomenon, which cannot only be explained by impaired NO production. Smoking is considered to be a typical model of increased oxygen radical stress.44 This has been demonstrated by the finding of a compromised endothelial function18,19,45 as well as observations that administration of scavengers of reactive oxygen species can improve NO bioavailability in hypercholesterolemia.41–43. Our data support the notion that in the early phase of atherosclerosis, ie, in the presence of hypercholesterolemia, decreased NO bioavailability is probably indeed a multifactorial phenomenon, which cannot only be explained by impaired NO production.

Smoking is considered to be a typical model of increased oxygen radical stress.44 This has been demonstrated by the finding of a compromised endothelial function18,19,45 as well as observations that administration of scavengers of reactive oxygen species can restore endothelial dysfunction.44,46 Our data support the concept that NO bioavailability is decreased in smokers mostly as a result of enhanced NO degradation, because we found no difference in NO production between smoking and nonsmoking healthy subjects (Table 2) nor between smoking and nonsmoking patients with CRF (data not shown). This also implies that smoking did not contribute to the impaired NO bioavailability in those patients with CRF who were smokers.

The LC-MS technique employed by us is capable of detecting relevant decreases in NO production, as demonstrated by the finding of a decrease in NO production from 0.30±0.14 to 0.10±0.06 μmol · kg⁻¹ · h⁻¹ in subjects receiving an intravenous infusion of the synthetic NOS inhibitor L-NMMA in a previous study.20 In addition, arginine fluxes and arginine-to-citrulline conversion rates, as measured in the healthy control groups, are of the same order of magnitude as values reported in the literature obtained by GC-MS and GC-IRMS techniques.17,47

A limitation inherent to all techniques used to measure NO production17,20,47 is that they do not discriminate between the various isoforms of NOS (endothelial, neuronal, and inducible). In animal studies, both renal failure and atherosclerosis have been associated with increased, as well as decreased, expression of iNOS, depending on the stage of disease.48–51 This makes it difficult to assess to what extent changes in iNOS expression contributes to our observation of reduced NO production in these humans. If iNOS is present in the renal patients, this would mean that eNOS activity in patients with CRF must be suppressed even to a larger degree than becomes apparent from overall NO production. On the other hand, even if the reduction in NO production in these patients was entirely caused by a reduction in iNOS, this may be relevant to atherosclerosis. Recent studies indicated that iNOS expression is important in prevention of neointima proliferation and endothelial regeneration,52–54 whereas iNOS blockade could accelerate atherogenesis, and vascular transfection of iNOS could inhibit atherogenesis.54,55 Another, more practical limitation is that the technique, being expensive and technically complicated, could not be applied to large groups of patients. Nevertheless, despite these limitations, the degree of impairment of NO production in our patients with CRF was found to be statistically significant and very consistent, which underscores the severity of the observed impairment in NO function as a mechanism for endothelial dysfunction in these patients.

Acknowledgments
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References
10. Reyes AA, Purkerson ML, Karl I, Klahr S. Dietary supplementation with L-arginine has been found in hypercholesterolemia. 34,35 On the other hand, many studies have suggested that hypercholesterolemia is accompanied by increased oxygen radical stress, which is an important determinant of NO bioavailability.36–39 In this respect, the reaction of NO with superoxide and the subsequent formation of peroxynitrite appears to be crucial in determining NO bioavailability.40 In various experimental studies increased endothelial superoxide generation associated with hypercholesterolemia has now been demonstrated.36–38 One major source of superoxide production in hypercholesterolemia appears to be xanthine oxidase; inhibitors of this enzyme reduced endothelial superoxide production in vitro40 and restored endothelial dysfunction in vivo.41 Another source is NOS itself, which may exhibit uncoupling of L-arginine oxidation.2 This is underscored by the observation that administration of scavengers of reactive oxygen species can improve NO bioavailability in hypercholesterolermia.41–43. Our data support the notion that in the early phase of atherosclerosis, ie, in the presence of hypercholesterolemia, decreased NO bioavailability is probably indeed a multifactorial phenomenon, which cannot only be explained by impaired NO production.
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