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Nitric oxide production is low in end-stage renal disease patients on peritoneal dialysis

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Abstract

To test the hypothesis that nitric oxide (NO) deficiency occurs in end-stage renal disease (ESRD), NO oxidation products ($\text{NO}_2 + \text{NO}_3 = \text{NO}_x$) and cGMP were measured in blood, urine, and dialysate effluent of peritoneal dialysis (PD) patients and compared with blood and urine of healthy subjects. All subjects were on a controlled low-nitrate diet ($\sim 330 \mu\text{mol/day}$). NO_x and cGMP outputs were significantly reduced in PD patients ($334 \pm 50 \mu\text{mol/24 h}$ and $55 \pm 13 \text{ nmol/24 h}$, respectively) vs. controls ($823 \pm 101 \mu\text{mol/24 h}$ and $149 \pm 46 \text{ nmol/24 h}$). Plasma arginine was borderline low, plasma citrulline was elevated and plasma levels of the endogenous NO synthase inhibitor asymmetric dimethylarginine were approximately five time higher in PD patients ($2.2 \pm 0.3 \mu\text{M}$) vs. controls ($0.4 \pm 0.1 \mu\text{M}$). Although blood pressure (BP) was not different between groups at the time of study, 10 of 11 PD patients were on medication for hypertension. These studies demonstrate that total NO production is low in ESRD, and with appropriate caution, we conclude that this NO deficiency may contribute to the increased BP that occurs in ESRD.

Keywords

hypertension; arginine; citrulline; guanosine 3',5'-cyclic monophosphate; endogenous nitric oxide synthase inhibitors

Chronic, experimentally induced nitric oxide (NO) deficiency in animals produces hypertension, and there is evidence that regional vascular endothelial NO production may be defective in some individuals with primary and secondary hypertension (3). Hypertension is prevalent in patients with end-stage renal disease (ESRD), irrespective of the etiology of their renal failure. Many ESRD patients are refractory to multiple antihypertensive agents even in conjunction with fluid restriction and maintenance dialysis fluid removal (15,16). NO deficiency has been reported in children and adults with chronic progressive renal disease (5, 8) and has been implicated in ESRD, based on indirect evidence (14,19). There have, however, been no clinical studies that directly assess the NO status in ESRD. Accordingly, we conducted

the present experiments to specifically test the hypothesis that NO deficiency occurs in peritoneal dialysis (PD)-dependent patients. Several indexes of the L-arginine:NO system were measured, including $\text{NO}_2 + \text{NO}_3$ (NO_x), the stable inert oxidation products of NO, the second messenger cGMP, as well as plasma levels of arginine, citrulline, and the endogenous NO synthase (NOS) inhibitors asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and *N*-monomethyl-L-arginine (L-NMA). Of importance, these experiments were conducted under conditions of controlled dietary NO_x intake, the only situation in which NO_x levels in body fluids can give insight into NO production (4).

METHODS

Informed consent to participate in the study for measurement of 24-h NO_x production was obtained from 11 PD patients and 11 normotensive, healthy subjects. Demographic information on both groups is given in Table 1. Criteria for controls were as follows: normotensive, healthy individuals with no known illnesses and taking no cardiovascular medications. Of the 11 PD patients, 6 had been diagnosed with diabetic nephropathy, 2 with hypertensive nephrosclerosis, and 1 each with glomerulonephritis, renovascular disease and obstructive uropathy. All but one of the PD patients were taking antihypertensive medication.

For 48 h, all participants consumed nutritionally complete (~2,000 calories/24 h), low NO_x content (330 μmol , i.e., ~15–30% of normal dietary NO_3 intake; Ref. 4) food which was also low in sodium (60 meq/day), and potassium and phosphorus restrictions appropriate for dialysis-dependent patients were also maintained. All participants received complete preweighed packages of food and reported any food not consumed. Control patients collected all urine (with boric acid as preservative), and PD patients collected all peritoneal dialysate effluent plus any urine made, during the second 24-h period of low dietary NO_x intake. Blood pressure (BP) was measured while subjects were quietly seated, and a blood sample was obtained at the end of the 48-h diet. Plasma for cGMP analysis was stored with the phosphodiesterase inhibitor IBMX to prevent breakdown of cGMP in vitro.

The following analyses were conducted: NO_x concentrations of plasma, urine, and peritoneal dialysate were measured using the Griess assay after conversion of NO_3 to NO_2 with the NO_3 reductase enzyme, as described previously by us (18). In preliminary studies, we obtained complete recovery of NO_3 added to PD fluid. Plasma and urine cGMP was measured by the Cayman Chemicals ELISA kit. Plasma arginine and citrulline were measured by reverse-phase HPLC with precolumn derivatization and fluorescent detection using a modification of the AccQ Tag system for amino acid analysis (Waters, Milford, MA). With α -aminobutyric acid as an internal standard, plasma samples were ultrafiltered with a 10,000 mol wt cutoff and then derivatized at 55°C with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (7). Dual pumps (Waters model 510) delivered the binary mobile phase (140 mM sodium acetate, with 17 mM tetraethylammonium titrated to pH 5.05; and 60% acetonitrile) at 1 ml/min using the AccQ Tag gradient table to a C_{18} column (Nova-Pak, 4 μm , 3.9 \times 150 mm, Waters), maintained at 60°C to allow resolution of citrulline and threonine. Ten-microliter samples were injected by autosampler (Waters model 717 plus) with the eluting products measured with a fluorescence detector (Waters model 474) at excitation and emission wavelengths of 250 and 395 nm (gain 100), respectively.

For measurement of the endogenous NOS inhibitors ADMA, SDMA, and L-NMA, the reverse-phase HPLC with AccQ Tag method of Anderstam et al. (1) was used with minor modifications. Standards of L-NMA, ADMA, and SDMA were run in the concentration range from 0.625 to 10.0 μM . Recoveries of spiked synthetic ADMA, SDMA, and L-NMA were ~100%. Concentrations of arginine, citrulline, and methylarginines were calculated using Millennium

chromatography manager (version 2.10) software for integrations and calculations based on the established standard curves for each run.

The methylarginine method was established by us after the NO_x production studies were completed, and therefore measurements were made on separate groups, all $n = 8$. All controls were a subset of the NO_x production series; PD group contained two of the original patients and six others, three with diabetic nephropathy, two with hypertensive nephrosclerosis, and one each with glomerulonephritis, membranous nephropathy, and scleroderma. Demographics are given in Table 1 in parentheses.

Plasma and urine sodium and potassium was measured by flame photometer, creatinine concentrations were by colorimetric assay of the Janovski complex (Sigma kit no. 555-A).

All data are reported as means \pm SE, and statistics were by paired and unpaired *t*-test and one way ANOVA. Statistical significance was defined where $P < 0.05$.

RESULTS

Table 1 summarizes baseline information on the subjects in both groups in the NO_x production and the ADMA studies. Generally, the groups were well matched, although the controls were younger than PD patients in the ADMA series and the gender mix was variable.

The 24-h output of NO_x in normal controls was significantly higher than in PD patients ($P < 0.001$) (Table 2). Dietary NO_x intake was similar in both groups, but the difference between output and intake, which provides a qualitative estimate of total NO production, was high in controls and lower, not different from zero, in PD patients. This does not mean that there was zero NO production in PD patients but rather provides a nonquantitative index of low NO production (8). The 24-h output of cGMP (the major second messenger of NO) was greater in controls than PD patients (Table 2, $P < 0.05$). The BP values for PD patients were not significantly different vs. controls but 10/11 PD patients were on antihypertensive medication at the time of study. Weight loss due to fluid removal averaged 1.20 ± 0.32 kg per 24-h treatment period in PD patients. PD patients had mean Kt/V values (i.e., urea clearance \times time of dialysis/volume of distribution) of 2.0 ± 0.2 per week, and the 24-h creatinine clearance in the control group was 129 ± 8 ml/min.

Plasma creatinine values were high in ESRD patients vs. controls as expected (Table 3). Plasma sodium was similar and plasma potassium was lower in PD patients vs. controls. Despite reduced production of NO_x and cGMP (indicated by low 24-h NO_x and cGMP outputs; Table 2), plasma concentrations of NO_x and cGMP were higher in PD vs. controls ($P < 0.001$), reflecting inadequate plasma clearance (Table 3). Plasma arginine values were reduced in PD patients ($P < 0.05$; Table 3), whereas plasma levels of citrulline ($P < 0.001$) and the endogenous NOS inhibitor ADMA were elevated approximately five times the control value in PD patients (Table 3). SDMA levels were also higher in ESRD vs. controls, but L-NMA was undetectable in most plasma samples, irrespective of group, and is therefore not reported.

DISCUSSION

The present study was designed to test the hypothesis that impaired NO production occurs in ESRD, using NO_x removal via urine and/or peritoneal dialysate as our index. We observed a marked reduction in the total rate of removal of the stable NO oxidation products, NO_x, in patients on PD, indicating that total NO production is low in ESRD. These measurements were made during controlled low dietary NO_x intake, a uniquely important aspect of this study. As discussed in detail by us elsewhere (4), dietary NO_x intake varies between subjects and within one individual from day to day. Since exogenous (dietary) NO_x and NO_x formed from

endogenously produced NO, cannot be differentiated in measurements of total NO_x. NO_x intake must be controlled when using NO_x levels to measure NO production.

The present studies indicate that total NO production is low in ESRD patients. The difference between NO_x intake and output was close to zero in ESRD, although presumably these patients do make some NO. Even subjects with normal renal function on a high NO_x intake excrete less NO_x in their urine than is ingested (4), reflecting other routes by which NO_x is lost from the body. Furthermore, we assume that the proportion of NO_x cleared from the body by PD, as a surrogate of functioning kidneys, is equivalent to that removed in the urine in controls, and this may not be correct. These considerations highlight the fact that NO_x output, from the kidney or surrogate, even when measured under ideal conditions, gives only a “qualitative” index of total NO production (4). Of note, it is unlikely that PD directly affected NO production, since inducible NOS is not stimulated by the native peritoneum, at least in the absence of peritonitis (17). One point that we cannot address in this study is whether total NO_x production necessarily reflects production of the “hemodynamically active” component of total body NO. The general assumption in the literature is that these variables are interchangeable, although there is no direct evidence to support this (4). There is widespread and heterogeneous tissue distribution of the various NOS isoforms, which will vary in disease (13); thus the relationship between total NO and “hemodynamically active” NO is likely to be variable and unpredictable.

Despite our finding from the NO_x output data that total NO production is reduced in ESRD, plasma NO_x levels, even after strict dietary nitrate restriction and an overnight fast, were higher in ESRD patients compared with controls. Since plasma NO_x is influenced by dietary NO_x intake as well as the time since eating and importantly, by renal clearance, plasma NO_x levels may reflect little about overall NO production (4). This is an important point, because investigators have begun to report that high plasma NO_x values automatically reflect increased systemic NO production, flawed conclusion.

Soluble guanylyl cyclase is an important receptor for NO (10), and cGMP levels often change in the same direction as changes in NO activity in the vasculature. In the PD patients, the decline in NO_x production was paralleled by a fall in cGMP, a reassuring finding. However, we caution against assuming a “cause-and-effect” relationship, since NO also signals by non-cGMP mechanisms, cGMP acts as second messenger for other agonists, and cGMP is removed from the body by a combination of excretion and metabolism.

What causes the deficiency in total NO production in ESRD? Arginine used for NO synthesis throughout the body is derived both from the diet and from that made endogenously (11,14). Lack of functional renal mass will compromise the main endogenous source of arginine generation and could lead to arginine deficiency and impaired NO synthesis. Indeed, experimentally induced arginine deficiency in rats is associated with decreased NO production and hypertension (20). Citrulline is the substrate for intrarenal arginine generation (11), and this accumulates in “anephric” patients (14). Citrulline levels were high in PD patients in the present study, which is suggestive of reduced renal arginine synthesis as well as reduced renal clearance.

Plasma arginine levels have been variously reported as normal or low in ESRD patients (14, 19) and in the present study were subnormal in PD patients. Given that clearance from the plasma is reduced as a result of the renal failure, low plasma arginine suggests a substantial reduction in available arginine in ESRD. However, the low plasma arginine levels in PD patients are still above the K_m of the various NOS enzymes (6) and should not lead to substrate-dependent falls in NO production, unless plasma arginine levels do not reflect intracellular arginine availability. In this regard, we recently reported that plasma from ESRD patients, and uremic levels of urea in artificial serum, inhibits arginine transport into cultured endothelial

cells (21,22). Therefore, there may be cellular arginine depletion in ESRD, and in support of this, orotic acid (a marker for relative arginine deficiency) is increased in renal failure (14).

There is also evidence that circulating levels of endogenous NOS inhibitors increase in renal failure (19). Of these NOS inhibitors, ADMA acts as a potent nonselective NOS inhibitor, whereas SDMA is ineffective (19). When plasma ADMA levels rise and arginine levels fall, NOS will be inhibited, if the rise in ADMA is of sufficient magnitude. According to Vallance and colleagues (19), plasma ADMA levels increase in uremia by about eight times, which brings the circulating ADMA concentration (~9 $\mu\text{mol/l}$) into a range where functional NOS inhibition is expected (19). Subsequently, other workers have also observed increases in plasma ADMA levels in ESRD, but report lower absolute values and smaller increases, such that plasma ADMA levels rise to 1.25 $\mu\text{mol/l}$ or less, a value which would have little impact on NOS activity (1,9,12). In the present study, we observed marked rises in plasma ADMA levels in PD patients to values that, although lower than originally reported (19), are sufficient to exert some NOS inhibitory effect. Therefore, although this area remains controversial, the present study supports the possibility that a rise in the ADMA: arginine ratio contributes to NO deficiency in patients with ESRD. In further support of our findings, NO deficiency has been reported in children and adults with chronic renal failure (5,8). Furthermore, chronic oral supplementation of L-arginine in rats with chronic renal failure and hypertension improved BP control, in addition to improvement of renal function (2).

In conclusion, although simple chemical indexes remain an imperfect measure of NO-dependent BP control, this carefully controlled clinical study provides evidence that total NO generation is substantially lower in ESRD patients treated for hypertension vs. normotensive controls. Production rate of the major second messenger, cGMP, also declines in uremic patients as does plasma substrate (arginine) levels, whereas the endogenous NOS inhibitor ADMA, increases. Low NO generation likely contributes to the hypertensive state in ESRD.

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Table 1
Characteristics of subjects in dietary controlled NO_x production study and in the endogenous NOS inhibitors study

| | Controls | PD Patients |
|--------------------|---------------|--|
| <i>n</i> | 11 (8) | 11 (8) |
| Sex, males/females | 5/8 (5/3) | 9/2 |
| Age, yr | 61±4 (47±5) | 69±4 [*] (72±3 [*]) |
| Body wt, kg | 74±2 (76±6) | 70±4 (69±6) |
| Height, cm | 170±2 (171±2) | 168±3 (165±3) |

Values are means ± SE; *n* = no. of subjects. Data in parentheses are for the endogenous nitric oxide synthase (NOS) inhibitors study. PD, peritoneal dialysis; NO_x, nitrate + nitrite.

^{*} *P* < 0.05, different vs. control.

Table 2
Summary of NO_x intake and output and cGMP output in control subjects and ESRD patients on PD

| | Control | PD Patients |
|-----------------------------------|---------|-------------|
| NO _x intake, μmol/24 h | 312±13 | 275±17 |
| NO _x output, μmol/24 h | 823±101 | 334±50 * |
| ΔNO _x , μmol/24 h | 525±108 | 57±26 * |
| cGMP output, nmol/24 h | 149±46 | 55±13 * |

Values are means ± SE. ESRD, end-stage renal disease; ΔNO_x, output - intake.

* $P < 0.05$ vs. control.

Table 3
Blood pressure and plasma concentrations in controls and in PD patients

| | Control | PD Patients |
|----------------------------------|-----------|------------------------|
| SBP, mmHg | 122±5 | 130±5 |
| DBP, mmHg | 78±4 | 78±2 |
| Plasma NO _x , μmol/ml | 28±3 | 41±5 [*] |
| Plasma cGMP, pmol/ml | 6±1 | 23±3 [*] |
| Plasma arginine, μM | 81±13 | 59±7 [*] |
| Plasma ADMA, μM | 0.40±0.08 | 2.16±0.27 [*] |
| Plasma SDMA, μM | 0.12±0.02 | 0.75±0.13 [*] |
| Plasma citrulline, μM | 26±2 | 60±7 [*] |
| Plasma creatinine, mg/dl | 0.8±0.1 | 8.8±0.7 [*] |
| BUN, mg/dl | | 47±4 |
| Plasma Na ⁺ , meq/l | 136±1 | 138±2 |
| Plasma K ⁺ , meq/l | 4.5±0.1 | 3.7±0.2 [*] |

Values are means ± SE. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; SBP, systolic blood pressure; DBP, diastolic blood pressure; BUN, blood urea nitrogen.

^{*} $P < 0.05$ vs. controls.