Decreased endothelial nitric oxide synthase in gastric mucosa of rats with chronic renal failure


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Tomikawa, M., M. Ohta, N. D. Vaziri, J. D. Kaunitz, R. Itani, Z. Ni, and A. S. Tarnawski. Decreased endothelial nitric oxide synthase in gastric mucosa of rats with chronic renal failure. Am. J. Physiol. 274 (Renal Physiol. 43): F1102–F1108, 1998.—According to recent reports, chronic renal failure (CRF) increases the susceptibility of gastric mucosa to injury. Since nitric oxide plays a major role in gastric mucosal defense and injury, we investigated, in rats with CRF produced by five-sixths nephrectomy and in control rats, the expression of nitric oxide synthase (NOS) in the stomach and measured mucosal and submucosal gastric blood flow. In CRF rats, gastric mucosal blood flow was significantly reduced compared with control rats, whereas submucosal and serosal blood flow was significantly increased. CRF significantly decreased endothelial NOS (eNOS) mRNA abundance by 53% (P < 0.01) and reduced expression of eNOS protein by 42% (P < 0.01) compared with the controls. Enzyme activity of eNOS was significantly reduced in gastric mucosa of CRF rats (P < 0.05). These data are consistent with reduced gastric mucosal blood flow in CRF rats and can explain altered susceptibility of gastric mucosa to injury in CRF rats.

MATERIALS AND METHODS

Animal models. Sixty male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) with an average body weight of 300 g were used. The animals were fed a standard laboratory diet (Purina Rat Chow; Purina Mills, Brentwood, MO) and water ad libitum. Animals were randomly assigned to the CRF and sham-operated control groups. Animals assigned to the CRF group underwent five-sixths nephrectomy. Under general anesthesia (with pentobarbital sodium, 50 mg/kg ip), the animals were subjected to a two-thirds left nephrectomy followed by a right nephrectomy 4 days later to produce CRF. The nephrectomies were carried out extraperitoneally through a dorsal incision. Systolic arterial blood pressure was monitored using a tail sphygmomanometer (Harvard Apparatus, South Natick, MA). At the end of the 6-wk observation period, the animals were placed in metabolic cages for a 24-h urine collection.
Urine was collected for the measurement of combined nitrate and nitrite (NO\textsubscript{2}/NO\textsubscript{3}) and creatinine. Plasma and urinary creatinine were measured using standard laboratory techniques. After measuring gastric blood flow, the animals were killed by decapitation, and stomachs were immediately harvested and frozen in liquid nitrogen or fixed in 10% Formalin or in 4% paraformaldehyde.

Measurements of urine NO\textsubscript{2}/NO\textsubscript{3}. The concentration of total NO\textsubscript{2}/NO\textsubscript{3} in the test samples was determined by a modification of the procedure described by Braman and Hendrix (4) using the purge system of an NO analyzer (N0A, model 270B; Sievers Instruments, Boulder, CO). Briefly, the urine samples were diluted 10 times in distilled water prior to analysis. A saturated solution of VCl\textsubscript{3} in 1 M HCl was prepared and filtered prior to use. A quantity of 5 ml of this reagent was purged with nitrogen gas for 5–10 min prior to use. To minimize foaming of the residual proteins in the samples, 100 µl of the 1/30 dilution of antifoam C (Sigma Chemical, St. Louis, MO) were added to the VCl\textsubscript{3} reagent. The purge vessel was equipped with a cold water condenser and a water jacket to permit heating of the reagent to 90°C, using a circulating water bath. The hydrochloric acid vapors were removed by a gas bubbler containing ~15 ml of 1 M NaOH. The gas flow rate into the chemiluminescence detector was controlled using a needle valve adjusted to yield a pressure of ~7 Torr. The flow rate of nitrogen into the purge vessel was adjusted to prevent vacuum distillation of the reagent.

Samples were injected into the purge vessel to react with the VCl\textsubscript{3}/HCl reagent, which converted NO\textsubscript{2}/NO\textsubscript{3}, and S-nitroso compounds to NO. The NO produced was stripped from the reaction chamber (by purging with nitrogen and vacuum) and detected by ozone-induced chemiluminescence in the chemiluminescence detector. The signal generated (NO peak and peak area) was recorded and processed by a Hewlett-Packard model 3390 integrator. In a typical assay, a quantity of 5 µl of the test sample was injected to the purge vessel, and all samples were run in triplicate.

Standard curves were constructed using various concentrations of NO\textsubscript{2} (5–100 µM) relating the luminescence produced to the given NO\textsubscript{2} concentrations of the standard solutions. The amount of NO\textsubscript{2}/NO\textsubscript{3} in the test sample was determined by interpolation of the result into the standard curve. The intra- and interassay variabilities for this assay were 4.59% and 5.91%, respectively.

Measurements of gastric blood flow. Gastric blood flow was measured under laparotomy in rats anesthetized with pentobarbital sodium (50 mg/kg ip). The measurements were performed using the laser-Doppler flow meter (model BLF 21; Transonic Systemic, Ithaca, NY) as previously described (13). The selected probe of the flowmeter with modification can measure submucosal blood flow including muscular and serosal blood flow, the fiber-optic probe (model HL-P1002) was applied gently to the gastric serosa. For the measurement of mucosal blood flow, the same probe was inserted through a small incision in the forestomach and applied gently to the gastric mucosa of the same gastric area, where serosal blood flow was measured. Blood flow measurements were performed with a time constant of 1.0 s and were recorded using a computer program (Windaq/200; Datalog Instruments, Akron, OH). A measurement was considered satisfactory when 1) it was stable for at least 10 s, 2) it was free of motion artifacts, and 3) the reading was reproducible. Ten measurements were performed in each animal. Gastric mucosal blood flow was expressed as tissue perfusion units (TPU) (3).

RNA isolation and RT-PCR for NOS. Gastric specimens for analysis of RNA were immediately frozen in liquid nitrogen, then stored at ~80°C. Frozen specimens were homogenized with a Polytron homogenizer (Kinematica, Littau, Switzerland) in 4 mol/l guanidinium isothiocyanate, and total RNA was isolated using the guanidinium isothiocyanate-phenol-chloroform method (5). The total RNA concentration in each sample was determined from absorbance at 260 nm, and the quality of each RNA preparation was determined by 1% agarose-formaldehyde gel electrophoresis and ethidium bromide staining.

RT-PCR were carried out using a GeneAmp RNA PCR kit and a DNA thermal cycler (Perkin-Elmer, Norwalk, CT) according to standard techniques (1). Briefly, 0.3 µg of total RNA was used as the template to synthesize complementary DNA with 2.5 U of Moloney murine leukemia virus reverse transcriptase, in 10 µl of buffer containing 10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 5 mmol/l MgCl\textsubscript{2}, 1 mmol/l of each deoxyribonucleoside triphosphate, 2.5 mmol/l of random hexamer, and 1.4 U/µl of ribonuclease inhibitor. RT was performed at room temperature for 20 min, then at 42°C for 15 min, at 94°C for 5 min, and at 5°C for 5 min. The resulting complementary DNA was used as a template for subsequent PCR.

The PCR specific primer set used for rat eNOS (24) was 5’ TACGGGACGACAAATCCAC 3’ (forward) and 5’ CAGGGTCGAGTCTTTGGAC 3’ (reverse). The PCR for β-actin was used as a positive control and an internal standard. The specific primer set for rat β-actin (rat β-actin control Amplifier set; Clontec Laboratories, Palo Alto, CA) (12) was 5’ TTGTAAACCAACTGGGACGATATGG 3’ (forward) and 5’ GATCTTGATCTTTGAGCTGAG 3’ (reverse). The PCR was performed in 50 µl of buffer containing 10 mmol/l Tris-HCl, pH 8.3, 2 mmol/l MgCl\textsubscript{2}, 50 mmol/l KCl, 0.2 mmol/l of each deoxyribonucleoside triphosphate, 0.25 µg of each primer, 2.5 µCi of [α-32P]dCTP, and 1.2 U of Taq DNA polymerase. To define the optimal amount of cDNA, increasing quantities of the RT cDNA products (2–12 µl) for both eNOS and β-actin were added to the PCR reaction (total 50 µl), and the resulting increase in PCR products was determined. This study demonstrated that the RT products were within the linear range of the PCR detection system. Therefore, the amplification of 10 µl of the RT cDNA product was used in this study. To define the optimal amplification cycle, another study was performed with 20–30 cycles of PCR amplification (27). Since the amplified products for both eNOS and β-actin during 24–30 cycles were also within the linear range of the PCR detection system, the amplification of 28 cycles was used in this study. The temperature profile of amplification consisted of 94°C for 1 min, 63°C for 1 min, and 72°C for 2 min.

Ten-microliter aliquots of PCR-amplified mixture were electrophoresed using a 1.25% agarose gel. The gel was dried using a gel dryer (model 543; Bio-Rad Laboratories, Hercules, CA) and subjected to autoradiography at room temperature for 3 h. The amplified cDNA products were identified by restriction enzyme analysis as reported in our previous study (13). The intensity of bands on the X-ray film was measured by densitometric scanning (Ultrascan XL laser densitometer; Pharmacia LKB Biotecnology, Uppsala, Sweden). The eNOS signal was standardized against β-actin signal for each sample, and results are expressed as the eNOS/β-actin ratio.

Western blot analysis. Frozen gastric specimens were homogenized with a Polytron homogenizer (Kinematica) in a lysis buffer containing 62.5 mmol/l EDTA, 50 mmol/l Tris, pH 8.0, 0.4% deoxycholic acid, 1% Nonidet P-40, 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin, 0.2 mmol/l...
pharmacophore with a Polyform homogenizer (Kinematica) in a buffer (250 mg/ml) containing 320 mmol/l sucrose, 0.1 mmol/l EDTA, 10 mmol/l HEPES, pH 7.4, 1 mmol/l DL-dithiothreitol, 10 µg/ml leupeptin, and 2 µg/ml aprotinin. The homogenates were then centrifuged (14,000 rpm for 20 min at 4°C). Such centrifugation removes tissue debris but not the membrane fraction. The protein normalized supernatants were assayed in duplicate.

The supernatant (40 µl) added to 100 ml of a buffer consisting of 40 mmol/l potassium phosphate, pH 7.4, 8 mmol/l L-valine, 1 mmol/l reduced nicotineamide adenine dinucleotide phosphate, 1 mmol/l MgCl2, 2 mmol/l CaCl2, 40 µmol/l L-arginine, 10 mg/l calmodulin, and 5.12 µCl/l L-[3H]arginine monohydrochloride (specific activity 64 Ci/mm; Amersham Life Science). Assays were also performed in the presence of 10 mmol/l L-NAME (L-NAME buffer) or in the presence of 10 mmol/l EGTA (EGTA buffer). Samples were incubated for 10 min at 37°C before termination of the reaction by the addition of 860 µl of H2O at 4°C. Then 250 µl of the diluted incubate solution was added to 250 µl of the Na+ form of Dowex 50W-X8 (Bio-Rad). The resin-incubate mix was settled for 15 min at 25°C and 100 µl of supernatant was pipetted into scintillation vials. Four milliliters of counting fluid (Cytoscint; ICN Pharmaceuticals, Costa Mesa, CA) were added to each vial. Measurements of radioactivity (in cpm), corresponding to [3H]citrulline, were made on a liquid scintillation counter (model LS580; Beckman Instruments, Palo Alto, CA). Results were expressed as picomoles per milligram of total protein per minute. The eNOS activity was determined from the difference between activities obtained in control and EGTA buffers, and the iNOS activity was determined from the difference between activities obtained in EGTA and L-NAME buffers.

# Results

General data and urinary excretion of NO metabolites. Weight gain and hematocrit in the CRF group were significantly lower, compared with the control rats, at the conclusion of the study (Table 1). A significantly rise in plasma creatinine and a significant decline in creatinine clearance were observed in the CRF group. Moreover, arterial blood pressure was significantly higher in CRF animals than in the control group. Urinary excretion of the NO metabolites, NO2/NO3, was significantly lower, compared with the control group.

**Table 1. General data and urinary excretion and plasma concentration of NO metabolites in CRF and control rats**

<table>
<thead>
<tr>
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<th>Control (n = 6)</th>
<th>CRF (n = 6)</th>
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<tbody>
<tr>
<td>Body wt, g</td>
<td>378 ± 5</td>
<td>346 ± 13*</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>125 ± 5</td>
<td>146 ± 4*</td>
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<tr>
<td>Hematocrit, %</td>
<td>46.4 ± 1.1</td>
<td>39.8 ± 0.8*</td>
</tr>
<tr>
<td>Pcreat, mg/dl</td>
<td>0.42 ± 0.03</td>
<td>1.33 ± 0.15*</td>
</tr>
<tr>
<td>Ccre, ml/min</td>
<td>2.62 ± 0.16</td>
<td>0.82 ± 0.14*</td>
</tr>
<tr>
<td>UNO2/NO3V, µmol/mg creatinine</td>
<td>0.49 ± 0.11</td>
<td>0.28 ± 0.06*</td>
</tr>
</tbody>
</table>

Values are means ± SD. CRF, chronic renal failure; MAP, mean arterial blood pressure; Pcreat, plasma creatinine; Ccre, creatinine clearance; UNO2/NO3V, urinary excretion of NO2/NO3. *P < 0.05 compared with the controls.
was significantly decreased by 43% in CRF rats (Table 1). The intra- and interassay variabilities for this assay were 4.59% and 5.91%, respectively.

Gastric blood flow. In CRF rats, gastric mucosal blood flow was significantly decreased (from 21.2 ± 2.8 in controls to 15.8 ± 4.6 TPU in CRF rats; \( P < 0.05 \)), whereas gastric submucosal blood flow was significantly increased in CRF rats compared with the control rats (from 11.5 ± 5.0 in controls to 24.8 ± 6.7 TPU in CRF rats; \( P < 0.05 \)) (Fig. 1).

Expression of eNOS mRNA in stomach. RT-PCR demonstrated expression of gastric eNOS mRNA in CRF and control groups (Fig. 2A). The abundance of eNOS mRNA in the CRF stomachs was reduced by 53%, compared with the controls (\( P < 0.01 \); Fig. 2B).

Expression and localization of eNOS and iNOS proteins in stomach. Western blots demonstrated the presence of eNOS (140-kDa bands) in stomachs of CRF and control groups (Fig. 3A). No iNOS protein (130-kDa bands) was detected by Western blots in the gastric mucosa of either normal or CRF rats (Fig. 3B). Gastric eNOS protein expression was reduced by 42% in the CRF group, compared with the controls (\( P < 0.01 \), Fig. 3C).

Immunofluorescence microscopy demonstrated that the distribution of eNOS was mostly localized to the endothelium of mucosal and submucosal vessels (Fig. 4, A and B).

The expression of eNOS fluorescence signal in the endothelium of mucosal and submucosal vessels was significantly reduced in CRF rats compared with the control rats (mucosal vein, 24% reduction; submucosal vein, 24% reduction; \( P < 0.01 \)) (Fig. 4C).

NOS enzyme activity. Gastric eNOS enzyme activity in the CRF rats was reduced by 21% compared with controls (\( P < 0.05 \)), whereas iNOS enzyme activity was similar in the two groups (Fig. 5).
DISCUSSION

This study demonstrated for the first time that eNOS mRNA abundance, protein expression, and enzyme activity are decreased in the stomachs of rats with CRF. Expression of eNOS protein signal detected by immunofluorescence staining was predominantly localized to the endothelium of mucosal and submucosal vessels and was significantly decreased in CRF rats compared with control rats. Although the methods used are semiquantitative, the results are internally consistent. Urinary excretion of NO\textsubscript{2}/NO\textsubscript{3} at steady-state condition was reduced in the CRF group, suggesting decreased total body NO production in these animals.

Since NO plays an important role in the maintenance of mucosal integrity by increasing mucosal blood flow (21), the decreased expression of eNOS may lead to decreased gastric mucosal flow and influence the susceptibility of the gastric mucosa to injury.

Utilizing direct measurements of mucosal blood flow, this study demonstrated that in CRF rats gastric mucosal blood flow is significantly reduced, whereas gastric serosal + submucosal blood flow is significantly increased compared with control rats. These new data, i.e., selectively reduced gastric mucosal blood flow, are consistent with and can be explained by reduced eNOS expression in gastric mucosa of rats. These data can also explain increased susceptibility of gastric mucosa of CRF rats to injury. The increased submucosal blood flow is consistent with submucosal blood shunting and/or development of arteriovenous malformations, which are present with increased frequency in clinical CRF. Quintero et al. (17, 18) have demonstrated increased basal gastric mucosal blood flow and increased susceptibility to gastric damage in CRF rats. Furthermore, they found that perfusion of stomach with acid further increased mucosal blood flow. This effect was abolished by specific NOS inhibitors, suggesting an NO-dependent mechanism governing the hyperemic response to luminal acid in CRF (18). They also found that systemic vascular resistance and peripheral blood flow were unchanged, and mean arterial pressure was increased (18). These findings indirectly suggest that CRF specifically affects gastric mucosal NOS activity. Since other examples of enhanced NO synthesis, such as sepsis and portal hypertension, are associated with systemic hyperdynamic circulation and decreased peripheral vascular resistance (16), the circulatory changes in CRF reported by Quintero et al. (17, 18) are much different from the above conditions.

In contrast to the indirect observations made by Quintero and co-workers (18), suggesting activation of gastric NO system, we found reduced eNOS mRNA abundance, protein expression, and enzyme activity in the stomachs of the CRF rats. Our data of gastric blood flow obtained with a selective measurements of blood flow in the mucosa and separately in the submucosa can explain differences between our studies and those of Quintero et al., because the indirect method used by Quintero et al. measures the blood flow in gastric wall (mucosa plus submucosa) rather than selectively in the mucosa. The laser-Doppler method used for the blood flow measurements in our study is based on the principle that light scattered by moving erythrocytes experiences a shift in its frequency. Thus this method measures directly flow of erythrocytes in the tissue. There are also several additional possible explanations for the increased submucosal blood flow in CRF rats. The negative effect of decreased NO production on gastric blood flow may have been offset by CRF-associated volume expansion, hypertension, and anemia, which tend to increase blood flow. It is also possible that other vasoactive dilatory mediators (e.g., adrenomedullin or atrial natriuretic peptide) are involved in the process of increased submucosal blood flow.

Fig. 4. Photomicrographs of immunofluorescence staining for eNOS in the gastric wall of control rats (A) and CRF rats (B). Staining is mainly localized to endothelium of mucosal and submucosal vessels. C: intensity of immunofluorescence signal for eNOS in stomachs of control rats (open bars) and CRF rats (solid bars); mv, endothelium of mucosal veins; m, muscularis mucosae; smv, endothelium of submucosal veins; pm, muscularis propria. Values are means ± SD in image intensity units (n = 6 animals per each group). *P < 0.01 compared with control rats.

Fig. 5. Enzyme activity of gastric eNOS (left) and iNOS (right). Values are means ± SD in pmol·mg of total protein·min\(^{-1}\) (n = 6 animals per each group). *P < 0.05 compared with control rats.
The increased submucosal blood flow could also be explained by "denervation hypersensitivity" to NO in CRF, in which low basal NO production may upregulate overall NO sensitivity, producing exaggerated physiological responses to stimuli (2). This phenomenon is suggested to be present in patients with essential hypertension (15), but its relevance to CRF remains speculative until definitively studied. It should be noted that in addition to NO, several other factors including hypervolemia, anemia, and hypertension frequently present in CRF, can affect local and systemic circulation. This can potentially account for a possible increase in submucosal gastric blood flow, despite depressed local NO production in the CRF animals shown here.

Another novel finding in this study was that only gastric eNOS expression was reduced, whereas iNOS expression was unaffected by CRF. There are few previous studies concerning the regulation of NOS activity in CRF. Noris and co-workers (11) demonstrated that platelets obtained from CRF subjects generated more NO than did platelets obtained from control subjects and that CRF plasma induced NO synthesis by normal platelets in vitro. On the basis of these data, the authors speculated that in CRF, iNOS activity was increased in response to elevated blood concentrations of tumor necrosis factor-α, which in turn were the result of decreased renal cytokine clearance in CRF (11). In contrast, Vaziri et al. (26) have shown marked downregulation of vascular and renal tissue NOS in CRF rats. The results of the present study demonstrating reduced NOS expression in gastric mucosa of CRF rats are consistent with those of Vaziri et al. (26) in the kidney and vascular tissue in this model.

Our previous study demonstrated that eNOS mRNA expression and enzyme activity in gastric mucosa are much higher than those of iNOS (13). This study did not demonstrate (by Western blot analysis) marked presence of iNOS protein in gastric mucosa of either normal or CRF rats. The iNOS activity assay, however, demonstrated low iNOS enzyme activity in both normal and CRF rats. These results are internally consistent, because the iNOS protein levels corresponding to such a low activities are below the range of detection levels by Western blot analysis.

In summary, rats with CRF have reduced expression of eNOS mRNA and protein, decreased eNOS enzyme activity in the stomach, and reduced gastric mucosal blood flow. These findings suggest that reduction of eNOS activity (by inference, reduced NO generation) causes reduced mucosal blood flow and affects susceptibility to gastric mucosal injury in CRF rats.

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