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.

The mortality from upper gastrointestinal hemorrhage is significantly increased in patients with chronic renal failure (CRF) compared with normal subjects (22). The propensity for upper gastrointestinal bleeding in patients with CRF has been primarily attributed to mucosal vascular malformations and peptic ulcer disease, which are both more prevalent in this population (28). Since CRF does not cause gastric acid hypersecretion (14), the impaired defense mechanisms are frequently implicated in the pathogenesis of nonvascular mucosal lesions in CRF. The CRF rat has been frequently used as a model system for evaluating gastric defense mechanism in CRF. Recent studies demonstrated that CRF rats have increased susceptibility to gastric mucosal acid-mediated damage in an aspirin injury model (17). The pathogenesis of these mucosal abnormalities has not been fully defined.

Nitric oxide (NO), a potent vasodilator, is generated from the terminal guanidino-nitrogen atoms of L-arginine by a family of enzymes known as NO synthase (NOS) (20). The role of NO in gastric mucosal defense and mucosal susceptibility to injury is complex and both increased and decreased NO generation have been linked to increased gastric mucosal susceptibility to injury. NO plays a major role in gastric mucosal defense by modulating the mucosal microcirculation (21). It has been demonstrated that endogenously generated or exogenously added NO protects the gastric mucosa against injury from ethanol or endotoxin-1, whereas inhibition of NOS increases susceptibility to gastric mucosal injury (8, 10). On the other hand, an excessive production of NO can be toxic to the gastric mucosa and can cause mucosal injury. In fact, we have demonstrated that the reversal of overexpressed NOS with N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) can mitigate gastric injury in rats with portal hypertension (13).

Effect of CRF on NO metabolism is controversial, with both increased and decreased NO synthesis reported in rats and humans (9, 11, 19, 25). Vallance et al. (25) showed that accumulation of endogenous N\textsuperscript{G}, N\textsuperscript{O}-dimethylarginine, an NOS inhibitor, may impair NO synthesis in CRF patients. Recently, Vaziri et al. (26) have demonstrated a marked downregulation of NOS expression in the remnant kidney and vascular tissues of rats with CRF. To our knowledge, the effect of CRF on gene expression, enzyme activity, or tissue localization of NOS in the stomach has not been investigated. Since, as stated above, marked changes of NO production can enhance the susceptibility of the gastric mucosa to injury (8, 10, 13), we postulated that increased gastric mucosal susceptibility to injury in CRF may be, in part, due to altered NOS expression and enzyme activity in this tissue. Therefore, we determined mRNA expression, protein expression, and NOS enzyme activity of both endothelial NOS (eNOS) and inducible NOS (iNOS), as well as eNOS protein localization in gastric mucosa. We also measured gastric blood flow.

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. Strict hemostasis and aseptic measures were observed during the surgical procedures. 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$_2$/NO$_3$) 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 gastric NO$_3$/NO$_2$. The concentration of total NO$_2$/NO$_3$ 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 (NQA, 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$_3$ in 1 M HCl was prepared and filtered prior to use. A quantity of 5 ml of each 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$_3$ 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$_3$/HCl reagent, which converted NO$_2$, NO$_3$-, 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$_2$ (5–100 µM) relating the luminescence produced to the given NO$_2$ concentrations of the standard solutions. The amount of NO$_2$/NO$_3$ 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 blood flow in the tissue to the depth of 0.5 mm. To 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 measurements of mucosal blood flow, the same probe was inserted through a small incision in the stomach 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; Datas 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 ~8°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$_2$, 1 mmol/l of each deoxyribonucleoside triphosphate, 2.5 mmol/l of random hexamer, and 1.4 U/ml of ribonuclease inhibitor. RT was performed at room temperature for 5 min, then at 42°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’-TACCGGAGCAGGAAATGGACACCC 3’ (forward) and 5’-CAGCTGCTCAGCTTGTGATGTCAGG 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 amplifier set; Clontech Laboratories, Palo Alto, CA) (12) was 5’-TTGTAACAACTGGGACGATATGG 3’ (forward) and 5’-GATCTTGATCTCTATGTCAGG 3’ (reverse). The PCR was performed in 50 µl of buffer containing 10 mmol/l Tris-HCl, pH 8.3, 2 mmol/l MgCl$_2$, 50 mmol/l KCl, 0.2 mmol/l of each deoxyribonucleoside triphosphate, 0.25 µg of each primer, 2.5 µCi of [γ-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 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 Biotecology, 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
phénylmethylsulfonyl fluoride, 0.05 mmol/l aminoethylbenzene sulfonyl fluoride. The homogenates were then centrifuged (14,000 rpm for 10 min at 4°C) to remove tissue debris without precipitating membrane fragments. The protein content of the homogenate was determined by the biouroninic acid protein assay (23), using a commercial kit (BCA protein assay reagent; Pierce Chemical, Rockford, IL). Proteins were eluted from the supernatant directly into SDS sample buffer and separated with 7.5% SDS-PAGE. Protein loading was equal (200 µg/well) in all lanes. Transfer blotting to nitrocellulose was performed in a buffer containing 25 mmol/l 3-[1-dimethyl-2-hydroxyethylamino]-2-hydroxypropanesulfonic acid, pH 9.5, in 20% methanol. Filters were blocked in a buffer containing 5% powdered nonfat milk, incubated for 1 h with specific polyclonal antibodies against eNOS (Affinity BioReagents, Neshanic Station, NJ) or iNOS (Transduction Laboratories, Lexington, KY), diluted 1:1,000, and incubated for 1 h with anti-rabbit IgG peroxidase conjugate (Sigma Chemical). The signal was visualized by the chemiluminescence method, using ECL Western blotting detection reagents and Hyperfilm-ECL (Amersham Life Science, Arlington Heights, IL). The lysate of endothelial cells (EA-hy 926; provided by Dr. Edgell, Department of Pathology, University of North Carolina, Chapel Hill, NC) (6) and the electrophoresis standard protein for iNOS (Cayman Chemical, Ann Arbor, MI), respectively, were used as positive controls for eNOS and iNOS. Quantification of protein signals was performed by densitometric scanning of autoradiographs (UltraScan XL Laser Densitometer, Pharmacia LKB Biotechnology).

Immunofluorescence staining for eNOS. Gastric specimens were fixed in 4% paraformaldehyde for 4 h and subsequently transferred to 0.5 mol/l sucrose in phosphate-buffered saline for 24 h. Then they were frozen at −80°C until cutting. Cryostat sections (10-µm thick, Jung Cryocut 1800; Leica, Deerfield, IL) were digested with 0.1% trypsin (Sigma Chemical) at 37°C for 10 min and incubated overnight with the specific polyclonal antibody against eNOS (Affinity BioReagents) diluted 1:100. This antibody was also used for Western blot analysis. For control studies, gastric sections were incubated overnight with phosphate-buffered saline instead of the primary antibody. After washing with phosphate-buffered saline, sections were incubated for 30 min with fluorescein-conjugated immunoglobulin (Sigma Chemical) diluted 1: 50. Immunofluorescence of coded sections was evaluated using a Nikon Optiphot epifluorescence microscope with B filter composition (Nikon, Garden City, NY). For the quantitative assessment of fluorescence intensity we used a Nikon TMD Diaphot microscope connected to a video analysis system (Image-1FL; Universal Imaging, Westchester, PA) (13). The Image-1 system allows an image to be entered into computer memory in a fraction of second, therefore fading of fluorescence is not a problem. This system distinguishes density of staining on a scale of 0–255 units. All measurements of fluorescence intensity were made by an investigator unaware of the code. Fluorescence intensity in the endothelia of mucosal veins (collecting venules) and submucosal veins was measured in standardized rectangles under ×400 magnification in 10 randomly selected fields of each section. Each measurement was standardized by subtracting the background intensity in each slide. All samples were processed and immunostained at the same time, and fluorescence intensity was measured on coded sections during the same session and under the same conditions.

NOS activity assay. NOS activity was measured by determining the conversion of L-[3H]arginine to [3H]citrulline according to the method described by Fernández et al. (7). Frozen gastric specimens obtained from operated rats were homogenized with a Polytron 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 d,l-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) was added to 100 µl of a buffer consisting of 40 mmol/l potassium phosphate, pH 7.4, 8 mmol/l L-valine, 1 mmol/l reduced nicotinamide adenine dinucleotide phosphate, 1 mmol/l MgCl2, 2 mmol/l CaCl2, 40 µmol/l l-arginine, 10 mg/l calmodulin, and 5.12 µCi/l [3H]arginine monohydrochloride (specific activity 64 Ci/mmoll; 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 NaHCO3 buffer. The resinlineucinate mix was settled for 15 min at 25°C. The supernatant was pipetted into scintillation vials. Four milliliters of counting fluid (Cytoscent; 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 LS5801; 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.

Statistical Analysis. Results are expressed as the means ± SD. Student’s t-test was used to compare data between CRF and control groups. One-way analysis of variance and Bonferroni correction were used to compare differences between localized fluorescence intensity in the immunostaining.

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 significant 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 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).

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**Fig. 1.** Gastric submucosal and mucosal blood flow. Values are means ± SD (n = 12 animals per each group); TPU, tissue perfusion units. *P < 0.05 compared with controls. Control, control rats; CRF, chronic renal failure rats.

**Fig. 2.** A: RT-PCR analysis for endothelial NO synthase (eNOS) mRNA and β-actin mRNA in gastric tissues. B: quantitative analysis of eNOS mRNA in the gastric tissues using densitometric scanning of amplified PCR products. Each eNOS signal was standardized against the corresponding β-actin signal, and results are expressed as eNOS/β-actin ratio. Values are means ± SD (n = 18 animals per each group). *P < 0.01 compared with controls.

**Fig. 3.** Western blot analysis for eNOS (A) and inducible NOS (iNOS; B) in gastric tissues. eNOS and iNOS proteins are expressed as 140- and 130-kDa bands, respectively. C: quantitative analysis of eNOS protein expression in the gastric tissues using densitometric scanning of Western blots. Values are means ± SD in densitometric units (n = 12 animals per each group). *P < 0.01 compared with controls. EC, lysate of endothelial cells (positive control); P, electrophoresis standard protein for iNOS (positive control).
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); m, muscularis mucosae; mv, endothelium of mucosal veins; 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\textsuperscript{-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 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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