Regulation of atrial natriuretic peptide gene expression in gastric antrum by fasting

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1Laboratory, 2Medicine and 3Surgery Services, James A. Haley Veterans Hospital, and Departments of 4Biochemistry and Molecular Biology, 5Internal Medicine, 6Physiology and Biophysics, and 7Surgery, University of South Florida, and 8University of South Florida Cardiac Hormone Center, Tampa, Florida 33612

Gower, William R., Jr., Khaled F. Salhab, Wendy L. Foulis, Nirmala Pillai, Jason R. Bundy, David L. Vesely, Peter J. Fabri, and John R. Dietz. Regulation of atrial natriuretic peptide gene expression in gastric antrum by fasting. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R770–R780, 2000.—Atrial natriuretic peptide (ANP) gene expression was localized in the rat gastric antrum using immunohistochemistry and in situ hybridization to mucosal cells in the lower portion of the antropyloric glands. Colocalization of immunoreactive ANP, long-acting natriuretic peptide, i.e., proANP-(1—30), and serotonin in these cells identified them to be enterochromaffin cells. Fasting for 72 h in 8-mo-old (adult) rats produced a significant (P < 0.05) decrease in the levels of ANP prohormone mRNA, immunoreactive proANP-(1—30) and ANP to ~33% of that of fed rats. Fasting in 1-mo-old rats had no effect on these parameters. Transcripts for natriuretic peptide receptor subtypes NPR-A, NPR-B, and NPR-C were found in both mucosa and muscle tissues of the antrum. ANP, brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) stimulated the production of cGMP in antral mucosa in vitro with a potency of ANP > BNP > CNP, suggesting that these receptors were functional. We conclude that fasting decreases ANP prohormone mRNA and its gene products, long-acting natriuretic peptide, and ANP in the antrum of adult rats.

atrial natriuretic peptide receptors; stomach; enterochromaffin cells; reverse transcription-polymerase chain reaction

ATRIAL NATRIURETIC PEPTIDE (ANP) is a member of a family of natriuretic peptides that includes brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and long-acting natriuretic peptide [proANP-(1—30)], consisting of the first 30 amino acids of the ANP prohormone (25). ANP regulates a variety of physiological functions, including natriuresis, diuresis, and vasodilation (25). Three types of natriuretic peptide receptors for ANP, BNP, and CNP have been identified. These are the natriuretic peptide receptor type A (NPR-A), type B (NPR-B), and type C (NPR-C) (5, 15). NPR-A has guanylate cyclase activity and mediates the biological functions of ANP through the synthesis of cGMP (5). NPR-A preferentially binds ANP and BNP, but has a low affinity for CNP. The rank order of the binding affinity and potency for cGMP production by the NPR-A receptor is ANP > BNP >> CNP (25). NPR-B is similar to NPR-A in that it has an intrinsic guanylate cyclase but has a much higher affinity for CNP than either ANP or BNP (5). The rank order of binding affinity and potency for cGMP production by the NPR-B receptor is CNP >> ANP = BNP (25). The NPR-C receptor has equal affinity for ANP, BNP, and CNP; lacks an intracellular guanylate cyclase domain; and functions as a clearance receptor for all three natriuretic peptides (15).

Although ANP is synthesized primarily in the heart and functions as a cardiac hormone, the fact that ANP and its receptors are coexpressed in numerous extracardiac tissues, e.g., lung, thymus, gastrointestinal (GI) tract, suggests a possible role as a regional regulator acting as an autocrine and/or paracrine regulatory peptide (5, 14, 25). Immunoreactive ANP and ANP prohormone mRNA are present in the GI tract of several species, including rat, guinea pig, and human (14). We and others (12, 27) demonstrated the presence of immunoreactive ANP and its prohormone, proANP-(1—126), in extracts of whole rat stomach. With the use of ribonuclease protection analysis and RIAs, ANP transcripts and proANP-(1—126), the main storage form of ANP in the heart, were found predominantly in the proximal stomach and antrum (12). However, the cells synthesizing ANP in gastric tissues have not been identified. Very little is known about the expression and function of NPRs in the stomach (14). The aims of the present study were to 1) identify the antral mucosal cells that synthesize ANP, 2) examine the regulation of antral ANP prohormone gene expression by feeding and fasting, and 3) determine if functional ANP receptors are coexpressed in the antrum.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were received at 3 wk (100–125 g) and 31 wk (450–500 g; Harlan Sprague Dawley, Indianapolis, IN) and were maintained with free access to water and standard laboratory rat chow for 7 days. Animals in both age groups were randomized into two groups. One

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group was fasted for 72 h with free access to water and the second group was allowed free access to both food and water for 72 h. All animals were killed by decapitation. These studies were approved by the Institutional Animal Care and Use Committee of the University of South Florida in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

RNA extraction. Immediately after death, cardiac, kidney, lung, liver, spleen, and stomach tissues were surgically excised, rinsed in sterile Hanks’ balanced salt solution (HBSS), then total RNA was isolated using RNAzol B (Tel-Test, Friendswood, TX), as described previously (12). Polyadenylated [poly(A)^+] RNA was purified by oligo(dexoxythymidine)-cellulose column chromatography (Collaborative Research, Bedford, MA). Because the rat stomach demonstrates a heterogeneous structure displaying significant regional differences in structure and function, we separated the stomach into three regions in our analyses: the proximal or cardia region, the midregion or fundus, and the distal region or antrum (Fig. 1). Mucosa and muscle layers of the antrum were prepared by blunt dissection in cold sterile HBSS. The concentration of RNA isolated was measured by ultraviolet spectrophotometry, and the quality of the RNA was verified by agarose gel electrophoresis and ethidium bromide staining.

Northern blot and ribonuclease protection analyses. For Northern blot analyses, total RNA (20 µg) or poly(A)^+ (10 µg) samples were denatured, separated on 1.2% agarose-formaldehyde gels, transferred to Hybond N+ nylon membranes (Amersham Life Science, Arlington Heights, IL), and then the membranes were baked at 80°C for 2 h. Prehybridization was for 1 h at 45°C in Hybrisol I (Oncor, Gaithersburg, MD). For detection of gastrin mRNA, membranes were hybridized overnight at 60°C in fresh Hybrisol I containing 10^6 cpm/ml of rat gastrin cRNA probe. A [α-^32^P]UTP-labeled 220-bp cRNA probe complementary to rat gastrin mRNA was synthesized using SP6 RNA polymerase transcribed from rat gastrin cDNA (generously provided by Dr. Stephen J. Brand, Massachusetts General Hospital, Boston, MA) in pGEM 4z linearized with Pvu II (9). Membranes were washed in 2× SSC, containing 0.1% SDS (1× SSC = 0.15 M NaCl and 0.015 M citrate) at 24°C for 15 min and then 3× 20 min at 60°C in 0.1× SSC containing 0.1% SDS (9). Hybridization probes for NPR-A, NPR-B, and NPR-C (generous gift from Dr. David Garbers, Howard Hughes Medical Institute, University of Texas, Dallas, TX), and NPR-C (generous gift from Dr. David Lowe, Genentech, San Francisco, CA) were full-length gel-purified rat cDNAs and were labeled by random priming with [α-^32^P]dexo-CTP (Promega, Madison, WI) (4, 7, 20). Hybridization and washes were performed as described previously (4, 7, 12). Autoradiographs were obtained from the membranes and were quantified by video densitometry with a UVP GDS8000 gel documentation system (UVP, Upland, CA), using one-dimensional video densitometry software. Gastrin mRNA was normalized to the relative amounts of ethidium bromide-stained 28S rRNA. Normalization of NPR mRNA signals was with signals obtained after hybridization with a 1.0-kb glyceraldehyde-3 phosphate dehydrogenase probe (Clonetech Laboratories, Palo Alto, CA). Ribonuclease protection analysis for rat ANP was as described previously (12). Briefly, an [α-^32^P]UTP-labeled 278-bp cRNA probe complementary to rat ANP mRNA (31) was synthesized using T7 RNA polymerase transcribed from rat ANP cDNA (generously provided by Dr. David G. Gardner, University of California, San Francisco, CA) in pGEM 4z. A 132-bp β-actin probe, synthesized from a template containing a 249-bp fragment of the β-actin gene (Ambion, Austin, TX), was included in the hybridization reactions as an internal control. Total RNA (5–40 µg) and gel-purified probes were denatured, allowed to hybridize overnight at 45°C, then digested with RNase One (Promega, Madison, WI). Protected fragments were precipitated and then fractionated on a 6% denaturing polyacrylamide gel and analyzed by autoradiography. Autoradiographic signals were quantified by video densitometry as described above and normalized to the relative amounts of β-actin.

RT-PCR. Except where designated, all reagents used for RT-PCR were purchased from Life Technologies (Gaithersburg, MD). With the use of 20 µg of total RNA, single-stranded cDNA was synthesized in a 20-µl reaction mixture containing 1× first strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl_2), 10 mM dithiothreitol, 0.5 mM dNTP, 40 U RNasin (Promega, Madison, WI), 0.5 µg oligo(dexoxythymidine)_12–18 primer and 200 U Superscript II RT. The reaction was incubated for 15 min at 25°C, then for 50 min at 42°C, and then heated to 70°C for 15 min. After cooling to 4°C, 3 U of RNase H was added and the reaction mixture was incubated for 20 min at 37°C. In all cases, the authenticity of the PCR products was confirmed by hybridization to genespecific cDNA probes and optimization of amplification conditions was performed using a pool of rat antrum cDNA (data not shown). Each cDNA–primer combination was optimized for the number of thermocycles used and the starting cDNA concentration to ensure that synthesis remained in the exponential phase. Each multiplex PCR reaction mixture contained varying amounts of cDNA depending upon the number of thermocycles used and the starting cDNA concentration to ensure that synthesis remained in the exponential phase. Each multiplex PCR reaction mixture contained varying amounts of cDNA depending upon the number of thermocycles used and the starting cDNA concentration to ensure that synthesis remained in the exponential phase. Each multiplex PCR reaction mixture contained varying amounts of cDNA depending upon the number of thermocycles used and the starting cDNA concentration to ensure that synthesis remained in the exponential phase. 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1 min, 94°C for 1 min, 60°C for 1.5 min for 28 cycles; NPR-A and NPR-B, 96°C for 5 min, 60°C for 1.5 min, 72°C for 1.5 min, 80°C (add Taq polymerase) for 1 min, 93°C for 1 min, 60°C for 1.5 min, 72°C for 2 min for 30 cycles; NPR-C, 96°C for 5 min, 57°C for 1.5 min, 72°C for 2 min, 80°C for 1 min (add Taq polymerase), 93°C for 1 min, 57°C for 1.5 min, 72°C for 2 min for 30 cycles. To reduce the competitive interference in the multiplex PCR by the internal control (β-MG and to ensure its amplification was exponential, we used the primer-dropping method described by Wong et al. (29). Primers for β-MG were added after 10 cycles for ANP, NPR-A, and NPR-B and after 15 cycles for NPR-C. PCR products were analyzed by ethidium bromide-stained agarose gel electrophoresis. Comparative quantitation was performed by video densitometry as described above and normalized to β-MG.

Immunocytochemistry. Freshly excised atria and antrum were fixed in Zamboni’s fixative and embedded in paraffin. Sections (4–6 µm) were deparaffinized, rehydrated, and treated with 0.3% hydrogen peroxide in methanol for 30 min at 25°C to block endogenous peroxidase activity. Sections were then incubated for 20 min at 25°C with dilution buffer (PBS containing 0.3% Triton X-100 and 4% normal goat serum). Sections were incubated at 4°C for 16–18 h with primary antibody: rabbit anti-rat ANP, 1:250 (IHC 9103, Peninsula Laboratories, Belmont, CA); rabbit anti-human proANP(1–30), 1:500 (IHC 9129, Peninsula Laboratories); or rabbit anti-serotonin, 1:1,000 (S-5545, Sigma Chemical, St. Louis, MO). The ANP antiserum recognizes the 28-amino acid carboxy terminal of the prohormone of ANP (proANP-[99–126]) as well as ANP. The proANP(1–30) antiserum recognizes the 30-amino acid fragment of the amino terminus of the ANP prohormone and exhibits no cross-reactivity with the carboxy terminus of the prohormone or ANP (11). After washing twice with PBS for 5 min, tissue sections were incubated at 37°C for 1 h with 3 µg/ml biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) in the dilution buffer and then washed and incubated at 37°C for 1 h with a streptavidin-biotin complex reagent containing horseradish peroxidase (Vector Laboratories) in dilution buffer. After washing two times with PBS in 5 min, sections were incubated with metal-enhanced 3,3-diaminobenzidene solution (Pierce, Rockford, IL) for 10–12 min. Sections were washed in distilled water and counterstained with Mayer’s hematoxylin. Control sections incubated with normal rabbit serum instead of primary antibody or primary antibody preabsorbed with excess immunogen (10 µg/ml) exhibited no staining.

In situ hybridization. The nonradioactive detection method was performed using a commercial assay (RPN 3300, Amersham Life Science). In brief, cryostat (12 µm) sections were fixed with 4% paraformaldehyde, treated with 0.1% Triton X-100, and incubated with proteinase K (100 µg/ml) at 37°C for 20 min. Sections were incubated with glycine (2 mg/ml) for 5 min at 25°C, acetic acid (20%) at 4°C for 15 s, washed in PBS, and then dehydrated. The preparation of the ANP cRNA probes was similar to the method used in ribonuclease protection analysis, except that fluorescein-11 UTP was used rather than [32P]UTP. Hybridization was performed at 55°C overnight with 1.2 µg/ml fluorescein-labeled antisense or sense ANP cRNA. The sections were washed in decreasing concentrations of SSC, terminating with a final concentration of 0.2X at 55°C, treated with ribonuclease A, washed in 100 mM Tris-HCl, pH 7.5, 400 mM NaCl, incubated with blocking solution, and then incubated with an antifluorescein alkaline phosphatase-conjugated antibody for 60 min at 25°C. Hybridization signals were visualized by nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate chromogen.

Antral peptide extraction and RIAs. Extracts of antrum were prepared by boiling washed and preweighed tissues for 10 min in 10 vol of 1 N acetic acid containing 0.1 mM phenylmethylsulfonyl fluoride, as described in detail previously (12). Gastrin (13), ANP, and proANP(1–30) (11) concentrations in the supernatants were determined by specific RIAs as previously described. Gastrin antiserum 3021178 recognizes all amidated carboxy terminal molecular species (13). ANP antiserum (Peninsula Laboratories) recognizes the carboxy terminal amino acids 99–126 of the prohormone, i.e., ANP (15). ProANP(1–30) antiserum (Peninsula Laboratories) recognizes the amino terminal amino acids 1–30 of the prohormone (11). Both antisera cross-react with the complete ANP prohormone within tissues, but neither recognized the prohormone within the circulation (12).

cGMP production in antral mucosa. The effects of rat ANP, BNP, CNP, and analog C-atrial natriuretic factor (C-ANF) on cGMP production in antral mucosa incubated in vitro were as described previously (23). C-ANF is rat (des-Gln18,Ser19, Gly61,Leu23,Gly23)-ANF, a specific agonist for NPR-C. The muscle layer was separated from the antral mucosa by blunt dissection, and then the mucosa was cut into small segments (1–2 mm). Antral mucosa segments (3 or 4) were incubated in tubes containing 1 ml of Krebs-Ringer bicarbonate medium supplemented with amino acids and vitamins and gassed with 95% O2 and 5% CO2. Segments were preincubated for 30 min at 37°C in 1 ml of Krebs-Ringer bicarbonate medium containing 500 µM IBMX and 1 µM phosphoramidon. ANP, BNP, CNP, or C-ANF was added to each tube in a final concentration of from 10−10 to 10−6 M and incubated for an additional 30 min at 37°C. In some experiments, the explants were pretreated with 10−3 M of the NPR-A antagonist antanin (28) (Bachem, King of Prussia, PA) for 5 min before the addition of 10−6 M ANP or BNP. After the incubation, tissue segments were collected by centrifugation at 600 g and then were homogenized in 0.1 N HCl. The homogenate was centrifuged at 30,000 g for 10 min at 4°C, and then the supernatant was extracted with water-saturated ether three times and the levels of cGMP were measured by RIA after acetylation (Amersham Life Science). The detection limit of the assay was 0.150 pmol/tube, and the EC50 was 1.85 pmol/tube. The tissue pellet was resuspended in 1 N NaOH, and protein was measured by the Bradford method (BioRad Laboratories, Hercules, CA) with bovine serum albumin as the standard.

Statistics. Data are presented as means ± SE. Unpaired Student's t-test was used to compare between two experimental groups. Significance of differences between more than two groups was analyzed by ANOVA, followed by Bonferroni correction of t-tests for multiple comparisons against the same control group. Differences were considered significant at P < 0.05.

RESULTS

Expression of ANP prohormone mRNA in the stomach. Previously we used ribonuclease protection analysis to detect and semiquantitate ANP prohormone mRNA in rat gastrointestinal tract (12). To increase sensitivity, we developed and used an RT-PCR assay for detection of ANP prohormone mRNA in rat tissues. The relative difference in the signal for the cDNA produced with exon primers was nearly identical to that determined by ribonuclease protection analysis of the same samples (Fig. 2). The proximal stomach and antrum had two- and threefold higher levels of ANP transcripts than the fundus, respectively (Fig. 2).
Identification of ANP-expressing cells in antrum. We previously reported the presence of the complete ANP prohormone in acid extracts of rat antrum using two separate RIAs that employ antisera directed to opposite ends of the proANP-(1—126) molecule (12). When these same two antisera, i.e., antisera to amino acids 1—30 of the prohormone (long-acting natriuretic peptide) and antisera to amino acids 99—126 of the prohormone [proANP-(99—126) or ANP], were used to immunostain rat antria, identical intense positive staining was observed (Fig. 3, A and B). In the present comparative study, immunostaining of rat antrum with both antisera produced an indistinguishable pattern of immunopositivity (Fig. 3, D, E, G, and H), suggesting that the ANP prohormone is present in the antral mucosa. The immunoreactive ANP- and proANP-(1—30)-containing cells were found in low numbers predominantly in the lower third of the antral pyloric glands. The morphology of the individual proANP-immunoreactive cells was variable, exhibiting round, pyramidal, and flask shapes. The general epithelial morphology of these cells was typically endocrine in appearance, with a connection to the antral lumen and the majority of immunoreactivity localized to the basal portion of the cell (Fig. 3H). Because of the likely endocrine nature of the cell and with the knowledge that ANP is expressed in adrenal chromaffin cells as well as enterochromaffin (EC) cells in human colon, serial sections were stained with antisera to ANP and serotonin (marker for EC cells) (10, 18). In consecutive serial sections immunostained for ANP and serotonin, a very similar pattern of positive staining was observed (Fig. 3, J and K). These results suggest that it is the EC cells in the antral mucosa that synthesize ANP. No positive staining for immunoreactive ANP or proANP-(1—30) was detected in cells within the lamina propria, submucosa, smooth muscle, or ganglion cells. Specific staining was absent when nonimmune rabbit serum was substituted for proANP antisera (Fig. 3C) or when the antisera were preabsorbed with their respective peptide immunogens (Fig. 3, F and I).

The localization of ANP-synthesizing cells was confirmed by colorimetric in situ hybridization. The specificity of hybridization produced by the proANP antisense cRNA probe was evaluated by incubation with cryosections of rat atria. Similar to the pattern observed after immunohistochemical staining, the antisense probe produced intense paranuclear cytoplasmic staining of atrial myocytes (Fig. 4A). No signal was detected when the ANP sense cRNA probe was used (Fig. 4B). In antrum, the ANP antisense probe produced intense cytoplasmic hybridization signals primarily in cells scattered throughout the basal portion of the glands (Fig. 4C). These cells, similar to the immunoreactive proANP-containing cells, were few in number and exhibited considerable variation in their morphology. Occasionally, weak positive staining was observed within smooth muscle cells, but no staining was detected in lamina propria, submucosa, or ganglion cells. Staining specificity was confirmed by the absence of any positive signal when the sense probe was substituted for the antisense cRNA (Fig. 4D).

Effect of fasting on antral ANP prohormone gene expression. To determine whether the expression of ANP in stomach antrum is altered in response to altered gastric luminal environment, we examined the steady-state ANP prohormone mRNA levels in antrums from rats that were allowed free access to food and/or fasted for 72 h (food deprived). Furthermore, to investigate the effect of age on the ANP prohormone gene response to fasting, we compared the effects of fasting to feeding ad libitum in young (1 mo) and adult (8 mo) rats. To monitor the endocrine status of the antrum, we measured the steady-state levels of gastrin mRNA and the concentration of immunoreactive gastrin in the antrums of fed and food-deprived animals. In accordance with previous findings (30), both the abundance of gastrin mRNA and the concentration of immunoreactive gastrin were significantly decreased in antrums of animals after 72 h of fasting (Fig. 5). Fasting reduced antral gastrin levels to approximately one-third of that observed in fed animals (1 mo, 29%, P < 0.05; 8 mo, 38%, P < 0.05) (Table 1). Mean antral gastrin concentration decreased significantly to 23% in 1-mo-old rats and 22% in 8-mo-old rats. There were no significant differences in the antral levels of either gastrin mRNA or immunoreactive gastrin between 1- and 8-mo-old rats.

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When 1-mo-old rats were fasted for 72 h, the abundance of ANP prohormone mRNA was not significantly altered compared with rats fed ad libitum (Fig. 6). Food deprivation did not significantly alter the antral concentrations of immunoreactive ANP or proANP-(1—30) in 1-mo-old rats (Table 1). In contrast, in 8-mo-old rats, ANP mRNA abundance was significantly (P < 0.05) depressed to 33% the level in fed rats. Food deprivation resulted in similar decreases in antral concentrations of immunoreactive ANP and proANP-(1—30) to 36 and 38% of fed values, respectively (Table 1). Thus food deprivation in older adult rats produced a significant decrease in antral ANP gene products similar in magnitude to that observed for gastrin.

NPR subtype gene expression in stomach. To examine the distribution of NPR subtype mRNAs in stomach, we devised a sensitive RT-PCR assay specific for each receptor subtype. Because all primer pairs were designed to span an intron, amplification from cDNA vs. genomic DNA sequences was readily distinguishable by the predicted PCR product. NPR-A (575 bp), NPR-B (645 bp), and NPR-C (541 bp) primers produced a single PCR product of the expected size (Fig. 7A). No PCR product was detected in the absence of cDNA (data not shown). To validate the NPR RT-PCR assays, we examined a variety of rat tissues that had been previously reported to express NPRs. Figure 7 shows a representative result from RT-PCR assay of total RNA extracted from ventricle, kidney, lung, liver, spleen, and the three anatomic regions of the rat stomach. Transcripts for NPR-A, NPR-B, and NPR-C were found in all tissues assayed, confirming the reports that the NPR genes are widely expressed in the rat (5, 22). It is important to note in this figure that more natriuretic peptide receptors NPR-A and NPR-B transcripts were present in the stomach than in the kidney, liver, lung, or spleen.
Interestingly, transcripts for all three receptor subtypes were detected in each of the histologically distinct regions of the stomach. The distribution of NPR transcripts in antrum was further examined using total RNA extracted from mucosa and muscle layers. Figure 7, B and C, demonstrate that NPR-A, NPR-B, and NPR-C genes are expressed in both tissue layers of the antrum. Transcripts for all three NPRs were twice as abundant in smooth muscle than in mucosa. Within both tissue layers the order of relative abundance was NPR-A > NPR-B > NPR-C.

To ascertain the nature of the NPR mRNA species in these tissues, we examined poly(A)$^+$ RNA for NPR transcripts by Northern blot analysis. In confirmation of the results from RT-PCR analysis, transcripts for all three NPR subtypes were detected by Northern blot analysis in both antral mucosa and smooth muscle tissues (Fig. 8). Both NPR-A and NPR-B transcripts were detected at ~4.2 kb, a size similar to that observed in other tissues (1, 4, 20). NPR-C hybridization signals were multiple, with bands appearing at 9.0–2.0 kb. A similar pattern of multiple bands for NPR-C has been reported in other tissues (1, 7).

Stimulation of cGMP in rat antral mucosa by natriuretic peptides. The existence of functional NPR-A and NPR-B receptors in antral mucosa, i.e., within the immediate vicinity of EC cells, has not been demonstrated previously. To determine if the NPRs are expressed at functionally relevant levels in antral mucosa, the ability of ANP, BNP, and CNP to stimulate cGMP production was examined in antral mucosa in vitro. As shown in Fig. 9A, ANP, BNP, and CNP induced a concentration-dependent accumulation of intracellular cGMP, with an order of potency of ANP > BNP > CNP. At $10^{-6}$ M, ANP and BNP increased the intracellular levels of cGMP approximately fourfold, from an average control level of 109 ± 24 to 467 ± 28 and 378 ± 30 fmol/mg, respectively ($P < 0.01$). In contrast, CNP at the same concentration produced a significant, but lower, twofold increase (235 ± 23 fmol/mg; $P < 0.05$) in cGMP levels compared with controls. Maximal intracellular cGMP levels were achieved within 5 min after exposure to ANP, BNP, and CNP (data not shown). As expected, the NPR-C-specific agonist C-ANF had no effect on cGMP levels at all concentrations tested. These findings are consistent with the presence of both functional NPR-A receptors activated by ANP and BNP and NPR-B receptors activated by CNP.

To confirm that the NPR-A receptor was mediating the effects of ANP and BNP in the antral mucosa, we determined the effect of the selective NPR-A antagonist, anantin, on the stimulation of cGMP production by both ANP and BNP (28). Anantin is a functional NPR-A antagonist and has no agonist activity (28). As shown in Fig. 9B, anantin had no significant effect on basal cGMP production. Anantin, however, signifi-
cantly blunted both ANP- and BNP-stimulated cGMP accumulation by 60%. This suggests that ANP and BNP are mediating cGMP production in these preparations via the NPR-A receptor. These results are consistent with the RT-PCR and Northern blot data of the present study and indicate the presence of both functional NPR-A and NPR-B receptors in antral mucosa.

**DISCUSSION**

We and others (12, 27) have provided evidence that the ANP gene may be expressed in the rat stomach. In the present study, we have demonstrated the presence of varying levels of ANP prohormone mRNA in extracts of the three histologically distinct regions (cardia, fundus, antrum) of the rat stomach. This selective distribution, with antrum and cardia having much higher levels than the fundus, suggests that ANP may have tissue-specific functions within the stomach.

Our earlier findings that demonstrated that the rat antrum contains proANP-(1—126) coupled with the present results that show similar immunostaining patterns are produced when antibodies are directed to either the amino or carboxy terminus of proANP-(1—126) support the idea that ANP is stored within antral mucosal cells as its prohormone. This suggests that the posttranslational processing of preproANP in antral EC cells may be similar to that found in other ANP-synthesizing cells, e.g., human colon EC cells, adrenal chromaffin cells, and cardiomyocytes in which the ANP prohormone is the main storage form within the cell (10, 18, 25). Due to the relative abundance of both ANP prohormone mRNA and its gene product proANP-(1—126) within the antrum, we sought to identify the cells within this region that synthesize this prohormone.

**Table 1. Effects of fasting and feeding on antral gastrin, ANP, and pro-ANP-(1—30) concentrations**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>1 mo Old Fed</th>
<th>1 mo Old Fasted</th>
<th>8 mo Old Fed</th>
<th>8 mo Old Fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrin, nmol/g</td>
<td>2.32 ± 0.16</td>
<td>0.54 ± 0.15*</td>
<td>2.14 ± 0.20</td>
<td>0.48 ± 0.13*</td>
</tr>
<tr>
<td>ANP, pmol/g</td>
<td>0.73 ± 0.11</td>
<td>0.75 ± 0.07</td>
<td>0.95 ± 0.16</td>
<td>0.35 ± 0.06*</td>
</tr>
<tr>
<td>pro-ANP-(1—30), pmol/g</td>
<td>1.08 ± 0.13</td>
<td>1.19 ± 0.08</td>
<td>1.17 ± 0.13</td>
<td>0.45 ± 0.05*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 animals in each condition. ANP, atrial natriuretic peptide. *P < 0.05 vs. fed.
that adrenal chromaffin cells and EC cells in human colon synthesize ANP (10, 18).

Immunoreactive ANP has been reported in immune-type cells in lymphatic nodules in the lamina propria and the submucosa in guinea pig and rat intestine (14). In the human stomach, on the other hand, immunoreactive ANP has not been found in the lamina propria or submucosa (6). Similar to the results of Ehrenreich et al. (6) for human stomach, we did not detect any immunopositive staining for ANP or long-acting natriuretic peptide, i.e., proANP-(1—30) in the lamina propria or submucosa in the stomach antrum of the rat. We also did not detect ANP prohormone mRNA by in situ hybridization in these areas.

EC cells are an abundant type of enteroendocrine cell that contain serotonin and occur throughout the GI tract (21). On the basis of differences in the ultrastructural appearance of the secretory granules, it has been suggested that EC cells are comprised of several subpopulations of endocrine cells, each of which manufactures and stores different peptides (21). EC cells are typically of the open-type enteroendocrine cell, with a large basolateral compartment in contact with the basal lamina and a narrow apical process that allows

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Fig. 7. Expression of natriuretic peptide receptor (NPR) subtype mRNAs in various rat tissues. Total RNA was isolated from designated tissues and subjected to RT-PCR analysis for NPR-A, NPR-B, NPR-C, and β-MG mRNAs as described in MATERIALS AND METHODS. Amplified cDNA was fractionated on a 1.5% agarose gel and stained with ethidium bromide. A: tissue distribution of mRNA for NPR-A, NPR-B, NPR-C, and β-MG in whole tissue extracts of ventricle, kidney, lung, liver, spleen, and 3 regions of stomach. β-MG served as an internal control in all amplifications. Results are representative from 2 independent analyses using total RNA from 2 separate animals. Semiquantitative analysis of the relative expressions of NPR-A and NPR-B (B) and NPR-C (C) in antral mucosa (lanes 1, 3, 5) and smooth muscle (lanes 2, 4, 6). Data are expressed as ratio of receptor subtype cDNA to β-MG and shown as means ± SE from 3 animals. *P < 0.05 vs. mucosa.

Fig. 8. Northern blot analysis of NPR subtype mRNAs in antrum and lung. Poly(A)^+ RNA (10 µg) isolated from antral mucosa, smooth muscle, and lung was subjected to Northern blot analysis using probes for rat NPR-A, NPR-B, and NPR-C mRNAs. Membranes were stripped and probed for GAPDH.
access to the lumen (21). Results from our present immunohistochemical studies demonstrate that at least some of the ANP-containing studies demonstrate that at least some of the ANP-containing cells are exposed to both basal lamina and lumen (Fig. 3H), similar to the known subset of EC cells (21). Interestingly, after an increase in intraluminal pressure, duodenal EC cells release serotonin into both interstitial and luminal compartments (8). This observation may be pertinent to ANP as it may also be released into the lumen of the stomach secondary to a rise in intraluminal pressure, because the ANP-containing EC cells extend into the gastric lumen and it is known that a small increase (3 mmHg) in intra-atrial pressure will release ANP (25).

Our results are in agreement and extend the observations of Li and Goy (16) and Rambotti et al. (19) that demonstrate the presence of NPR-A and NPR-B transcripts in extracts of gastric fundus and localize natriuretic peptide-induced cGMP production to parietal cells, mucus secreting cells in the fundus, and pyloric glands, as well as gastric smooth muscle cells. Because ANP is known to stimulate gastric acid secretion and relax gastric smooth muscle (14) these findings suggest that these effects of ANP may be direct. Further evidence for functional receptors in gastric tissues comes from the reports that ANP stimulates the production of cGMP in guinea pig chief cells and that ANP induces the relaxation of cultured gastric smooth muscle cells (2, 3). Rambotti et al. (19) demonstrated the presence of ANP-induced guanylate cyclase activity on both apical and basolateral surfaces of mucosal cells within the pyloric glands of rat stomach, consistent with our suggestion that ANP released locally into the gastric lumen could target these luminally directed receptors. This suggests that ANP may help control a "negative feedback" system within the stomach of increasing acid secretion and simultaneously enhance mucus production to protect the lining of the stomach from the effects of acid. This would provide for a regulatory mechanism to ensure that the acid produced after a meal does not injure the mucosal surface of the stomach.

A very interesting finding of the present investigation was that fasting decreases ANP prohormone gene expression in adult rats. The present data are the first evidence that any change in the diet can alter ANP gene expression in the GI tract. The decrease in ANP gene expression with fasting is what one would expect to occur if the products of this gene, i.e., ANP and long-acting natriuretic peptide are important in helping to regulate fluid fluxes and peristalsis in the GI tract, as has been suggested previously (25). If food is not reaching the stomach, one would expect an increase in fluid absorption and decreased peristalsis if the stomach were helping to control distal GI tract function via its ANP hormonal system. Because the stomach is where digestion begins in the GI tract, one ideally would like to have a hormonal system based in the stomach that could communicate to the rest of the GI tract when to decrease its function when no food is entering the GI tract. The present investigation suggests that this is exactly what is occurring. Thus, with fasting, the expression of the ANP is markedly diminished, resulting in a marked decrease in the gene products, ANP and long-acting natriuretic peptide, that are available to inform the rest of the GI tract to decrease peristalsis and allow an increase in fluid absorption. With respect to this point, after eating, ANP increases 45% in the circulation (24), suggesting that feeding also regulates the release of ANPs. The reason why ANP prohormone gene expression was not suppressed by fasting for 72 h in 1-mo-old rats is not known at present, but suggests that the hormonal system is not fully developed in very young rats. The finding that the decrease in ANP expression in the antrum of food-deprived rats parallels the decrease in gastrin gene expression is also intriguing. Age-depen-

Fig. 9. A: accumulation of cGMP in rat antral mucosa after incubation with varying concentrations of ANP, BNP, CNP, and C-atrial natriuretic factor (C-ANF) in vitro. Antral mucosa segments were incubated in modified Krebs-Ringer bicarbonate medium containing 500 µM IBMX and 1 µM phosphoramidon without (control) and with varying concentrations of rat ANP, BNP, CNP, or C-ANF for 30 min at 37°C, then extracts were prepared for measurement of intracellular cGMP and protein. Data are means ± SE, n = 4. *P < 0.05 vs. control; **P < 0.01 vs. control. B: effect of NPR-A antagonist anantin on ANP- and BNP-induced intracellular formation of cGMP in antral mucosa in vitro. Antral explants were pretreated with anantin (10⁻⁶ M) for 5 min, and then incubated with either ANP (10⁻⁶ M) or BNP (10⁻⁶ M) for 30 min at 37°C in the presence of 500 µM IBMX. Antral explants were extracted, and then cGMP and protein content were determined. These are representative results from 2 independent experiments. Data are means ± SE, n = 4. **P < 0.01 vs. absence of anantin.
dent alterations in tissue concentrations, secretion rate, and response to luminal stimuli have been documented for other gastric regulatory peptides (14).

In conclusion, our results demonstrate that immunoreactive ANP, long-acting natriuretic peptide, and functional NPR-A receptors coexist in rat antral mucosa, suggesting a regional gastric ANP system whose gene expression and gene products are modulated by fasting.

Perspectives

The ability to decrease ANP gene expression and gene products in the stomach by fasting, with the corollary that these gene products increase in the circulation (24) and their excretion into urine increases (26) with food intake, suggests that distension or stretch of the stomach may be important to physiological regulation of the natriuretic peptide system within the GI tract similar to stretch of the heart being important for regulation of the same gene within the heart (14, 25). The present findings lead one to speculate that stretch throughout the GI tract may be an important regulator of this natriuretic peptide system. Because ANPs are present within the small intestine and colon (12, 14), as well as the stomach, as food moves through the small intestine and colon and, thereby, stretches the small intestine and colon, this may 1) stimulate ANP prohormone gene expression and/or 2) enhance release of its gene products to assist in the coordination of fluid homeostasis and motility throughout the GI tract.

The finding of the present investigation that ANP was localized near the basolateral surface of the EC cells suggests a mechanism for the previous observation that ANP increases in the circulation secondary to food intake. This basolateral plasma membrane is juxtaposed to capillaries via which atrial peptides could enter the circulation from the stomach.

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