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EXPRESSION OF ADRENOMEDULLIN IN PORTAL HYPERTENSIVE GASTRIC MUCOSA OF RATS

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We examined the expression and localization of adrenomedullin (AM) mRNA, AM receptor (AM-R) mRNA and AM protein in normal and portal hypertensive (PHT) rat gastric mucosa. Methods included competitive reverse transcription — polymerase chain reaction (RT/PCR), in situ hybridization and Western blot analysis. Both AM mRNA and AM-R mRNA were strongly expressed not only in normal rat gastric mucosa but also PHT rat gastric mucosa. While total AM and AM-R mRNA abundance was similar in both normal and PHT stomachs, in the superficial mucosa — where major defense mechanisms are located — their expression was significantly reduced in PHT rats by 33% (AM mRNA) and 25% (AM-R mRNA) (both $p < 0.05$). AM protein was also expressed similarly in both normal and PHT gastric mucosa. We conclude that AM and its receptor play a role in regulation of gastric mucosal microcirculation and thus gastric mucosal defense.

Key words: *adrenomedullin, gastric mucosa, portal hypertension.*

INTRODUCTION

Adrenomedullin (AM) is a potent vasoactive peptide which was first extracted from human pheochromocytoma tissue in 1993 (1). It is expressed in various tissues such as adrenal glands, lungs, kidneys, heart, spleen, duodenum and submandibular glands (2). AM, which has 25% homology with calcitonin gene-related peptide and islet amyloid polypeptide (amylin), consists of 52 amino acids, has one intramolecular disulfide bond and an amidated C-terminal tyrosine (1). AM has recently been shown to be synthesized and secreted by endothelial cells and vascular smooth muscle cells (3, 4), which also possess abundant AM receptors (AM-R) (5, 6). Therefore, it is suggested that

AM may participate in the local control of vascular tone as an autocrine/paracrine factor (7).

The gastric mucosal microcirculation, by delivering oxygen and nutrients to all mucosal constituents, plays an important role in mucosal defense to injury (8). It has been demonstrated that AM mRNA is expressed in normal gastric mucosa (9), and therefore may play a role in gastric mucosal microcirculation.

The portal hypertensive (PHT) gastric mucosa has increased susceptibility to damage by noxious factors such as alcohol, aspirin, and ischemia-reperfusion compared with normal mucosa (10—14). The mechanism of this increased susceptibility is not clear, but the abnormal microvasculature and microcirculation of PHT gastric mucosa may be the basis for this phenomenon (11, 15, 16). These microcirculatory abnormalities may be associated with and mediated by overexpression of vasoactive mediators, as has been demonstrated for nitric oxide and endothelin (17, 18). Therefore, AM — a potent vasoactive peptide — may also play an important role in the microcirculatory abnormalities of PHT gastric mucosa. A recent clinical study indicates that circulating levels of AM are increased in cirrhotic patients with ascities and correlate with hemodynamic abnormalities (19). The expression of AM and AM-R in PHT gastric mucosa, however has not been studied, forming the basis of this investigation.

MATERIALS AND METHODS

Animals Preparation

This study was approved by the Subcommittee for Animal Studies of the Long Beach (CA) Department of Veterans Affairs Medical Center. Forty-eight Sprague-Dawley rats (weighing 250—300 g) were used in the experiments. Rats were kept individually in wire bottom cages with free access to a standard rat chow (Rodent Diet No. 8504; Harlan Tekland, Madison, WI) and water. The animal room was illuminated on a 12 hours light-dark cycle. Room temperature was kept at 18—22°C and the humidity at 60—70%.

Twenty-four rats were anesthetized with Nembutal (50 mg/kg intraperitoneally) (Abbott Laboratories Ltd., North Chicago, IL). Portal hypertension was produced by staged portal vein occlusion and splenic vein ligation as previously described (10). Twenty-four sham operated (SO) rats (controls) underwent a similar operation but without occluding the portal and splenic veins. After 14 days stomachs of rats were excised and then frozen in liquid nitrogen and stored at −80°C for reverse transcription — polymerase chain reaction (RT/PCR), in situ hybridization and Western blot analysis.

RNA isolation and competitive RT/PCR

Frozen specimens were homogenized with a Polytron homogenizer (Kinematica AG, Littau, Switzerland) in 4 mol/L guanidinium isothiocyanate, and total RNA was isolated using the guanidinium isothiocyanate-phenol-chloroform method (20).

Reverse transcription was carried out using a GeneAmp RNA polymerase chain reaction kit and a DNA thermal cycler (Perkin Elmer, Norwalk, CT) as previously described (17). The resulting complementary DNA (cDNA) was precipitated and resuspended into 3 μg/μl.

The competitor was constructed to be used as a competitive internal standard in PCR amplification for quantitation of mRNA level of target genes, using the MIMIC™ construction kit (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instructions. Briefly, using the commercially synthesized primers (GIBCO BRL, Gaithersburg, MD), PCR was performed with the MIMIC DNA fragment provided in the kit to create PCR templates with AM-gene and AM-R gene specific 5' and 3' ends. These amplified templates were quantified and diluted before being used as competitive internal standards in PCR amplification.

Polymerase chain reaction was performed with 6 μg of cDNA (2 μl) and serial dilutions of the competitor (2 μl) ranging from 5.00×10^{-2} to 1.56×10^{-3} attomole/μl 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 each of deoxyribonucleoside triphosphate; 0.4 μmol/L of each primer; and 2 units of *Taq* DNA polymerase. For rat AM, the amplification was performed for 40 cycles of 1 minute each at 94°C for denaturing, 1 minute at 57°C for annealing and 2 minutes at 72°C for extension. The specific primer set used for rat AM was

5'-AAGAAGTGGAATAAGTGGGCG-3' (forward)

and

5'-TGTAAACTGGTAGATCTGGT-3' (reverse).

For rat AM-R, the amplification was performed for 40 cycles of 1 minute each at 94°C for denaturing, 1 minute at 63°C for annealing and 2 minutes at 72°C for extention. The specific primer set used for rat AM-R was

5'-AGCGCCACCAGCACCGAATACG-3' (forward)

and

5'-AGAGGATGGGGTTGGCGACACAGT-3' (reverse).

Nine -microliter aliquots of the products were subjected to electrophoresis on a 1.25% agarose gel and DNA was visualized by ethidium bromide staining. Location of the products (base pairs) was determined by using a 100-base pair ladder (GIBCO BRL) as standard size marker. The gel was then photographed under ultraviolet transillumination. The quantitative assessments of the PCR products were performed using a video analysis system (Image-1/FL, Universal Imaging Corp., Westchester, PA) (21). The Image-1 system can distinguish density on a scale of 0—255 units. Each measurement was standardized by subtracting the background intensity in average.

For comparison between SO and PHT stomachs, one MIMIC dilution was selected and all samples were processed at the same time.

In situ hybridization

The antisense and sense single strand cDNA probes for AM and AM-R were generated with RT/PCR. Briefly, using antisense or sense primers, RT/PCRs for AM and AM-R were performed with the buffer containing digoxigenin labeled dNTPs (Boehringer Mannheim, Indianapolis. IN). The products of initial RT/PCR were purified with a gel (GeneClean II kit, Bio 101, Inc., Vista, CA) and were used in the subsequent RT/PCR as templates. Hybridization was performed with Cryostat sections (10-μm thick; Jung CRYOCUT 1800, Leica Inc., Deerfield, IL) in a moist chamber at 42°C overnight. After stringency washes, digoxigenin was visualized using the Digoxigenin detection kit (Boehringer Mannheim). As a control, sense strand and RNAse A-treated sections were used for hybridization. All samples were processed and stained at the same

time. The intensity of staining was evaluated under 100 × magnification in 10 randomly selected mucosal fields of each section. The mucosal fields were classified into 3 areas, such as superficial mucosa, mucous neck, and basal mucosa. Each area was assessed using a score of 0—3 (0: no positive cells; 1: positive cells < 10% of all cells in the area, 2: positive cells 11%—50% of all cells in the area, 3: > 51% positive cells). All sections were coded and examined by 2 investigators unaware of the code.

Western blot analysis

Frozen gastric specimens were homogenized with a Polytron homogenizer (Kinematica AG) in NOVEX NuPAGE SDS Sample Buffer (Novel Experimental Technology (NOVEX), San Diego, CA) containing 0.5 μg/ml leupeptin; 0.5 μg/ml pepstatin; 0.5 μg/ml aprotinin; 0.2 mmol/L phenylmethylsulfonyl fluoride; 0.05 mmol/L aminoethyl benzene sulfonyl fluoride. The homogenates were then centrifuged (14.000 rpm for 10 minutes at 4°C). The protein content of the homogenate was determined by the bicinchoninic acid protein assay (22), using a commercial kit (BCA Protein Assay Reagent, Pierce Chemical Company, Rockford, IL).

Proteins were subjected to 10% Bis-Tris gel electrophoresis with NuPAGE MES SDS Running Buffer (NOVEX) and transferred to nitrocellulose in NuPAGE Transfer Buffer (NOVEX) containing 10% methanol. Filters were blocked in a buffer containing 1% bovine serum albumin, and incubated with the specific polyclonal antibody against rat adrenomedullin (Peninsula Laboratories, INC., Bermont, CA) diluted 1 : 1000 overnight at 4°C and incubated with anti-rabbit IgG peroxidase conjugate (Sigma Chemical Co., St. Louis, MO) for 1 hour. The signal was visualized by the chemoluminescence method (23), using ECL Western blotting detection reagents and Hyperfilm-ECL (Amersham Life Science Inc., Arlington Heights, IL). The protein extracted from rat adrenal glands was processed at the same time as a positive control. Quantitation of AM protein signals was performed by densitometric scanning of autoradiographs (Ultroscan XL Laser Densitometer; Pharmatica LKB Biotechnology).

Statistical Analysis

Results are expressed as the mean ± S.D. Student's t-test was used to compare data between SO and PHT rats. A p value less than 0.05 was considered statistically significant.

RESULTS

Expression of AM mRNA and AM-R mRNA

Competitive RT/PCR demonstrated expression of AM mRNA as well as AM-R mRNA in rat stomachs. An example of the ethidium bromide staining pattern of PCR products for AM is shown in *Fig. 1A*. The initial amount of AM cDNA can be estimated equal to that of MIMC, when both products after PCR are in equal amount. Accordingly, using 6.25×10^{-3} attomoles/ml of MIMIC, expression of AM mRNA was compared between SO and PHT stomach of rats (*Fig. 1B*). An example of the ethidium bromide staining pattern of PCR products for AM-R is shown in *Fig. 1C*. Using 1.25×10^{-2} attomoles/ml of MIMIC, expression of AM-R mRNA was compared between SO and PHT stomach of rats (*Fig. 1D*). There were no statistically significant differences in total expression of AM and AM-R mRNAs between these two groups (*Table 1*).

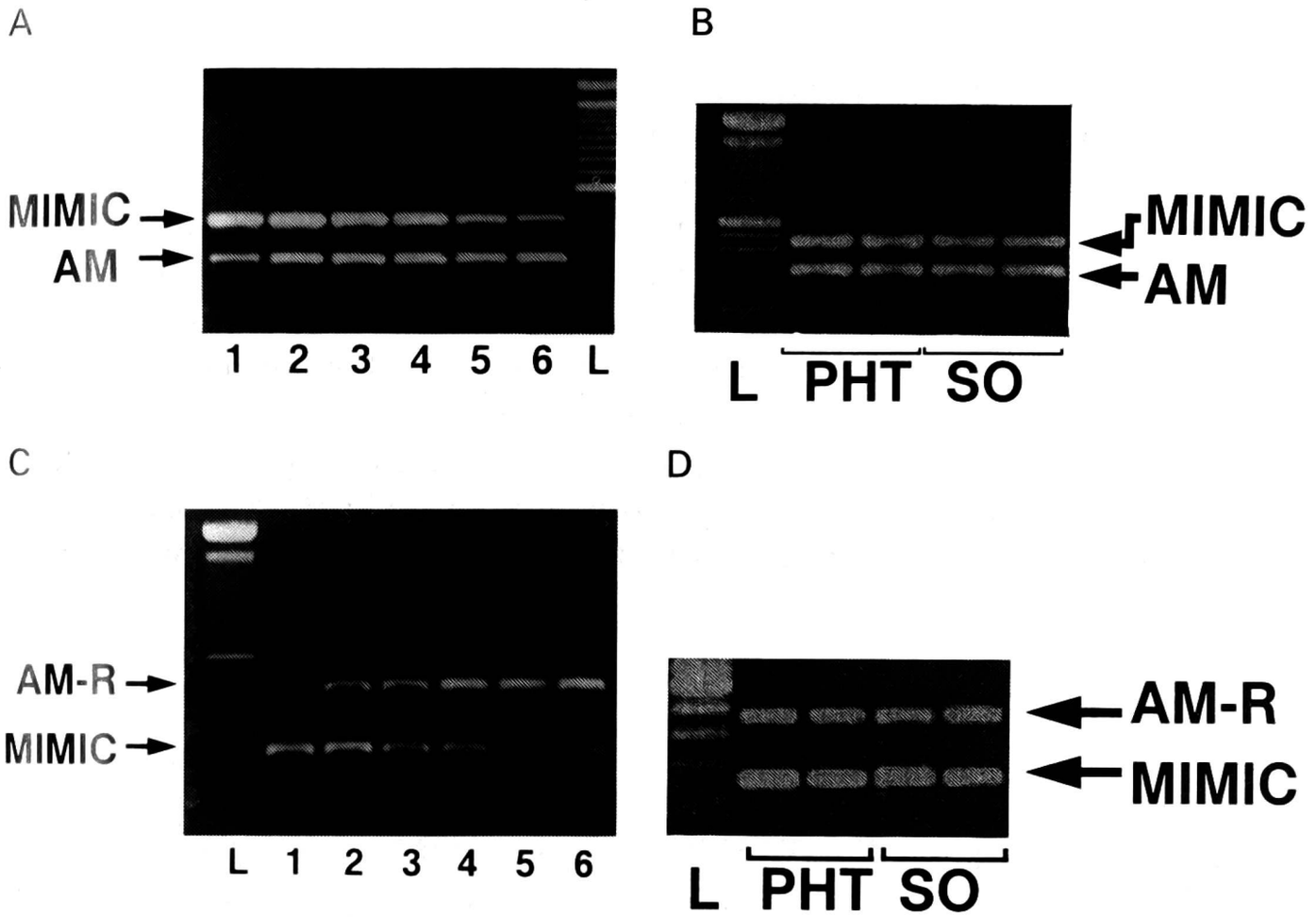

Fig. 1. Competitive RT-PCR analysis of both AM mRNA and AM-R mRNA in rat gastric mucosa. Ethidium bromide staining on 1.25% agarose gels for AM (A) or AM-R (C) and serial dilution of MIMICs. The sizes of AM and AM MIMIC products were 281 and 451 bp, respectively. The sizes of AM-R and AM-R MIMIC products were 470 and 250 bp, respectively. L: 100 bp ladder. Lane 1 contains 5.00×10^{-2} attomole/ml of MIMIC; lane 2: 2.50×10^{-2}; lane 3: 1.25×10^{-2}; lane 4: 6.25×10^{-3}; lane 5: 3.13×10^{-3}; lane 6: 1.56×10^{-3}. Expression of AM (B) and AM-R (D) mRNAs were compared between SO and PHT stomach of rats, using 6.25×10^{-3} and 1.25×10^{-2} attomoles/ml of MIMIC, respectively. L: 100 bp ladder, PHT: portal hypertensive rats, SO: sham operated rats.

Table 1. Expression and Localization of AM mRNA and AM-R mRNA in Gastric Mucosa of SO and PHT Rats.

	AM		AM-R	
	SO	PHT	SO	PHT
Total expression				
AM/MIMIC ratio	0.95±0.07	0.96±0.09	—	
AM-R/MIMIC ratio	—		0.82±0.08	0.79±0.06
Localization (score)				
Superficial mucosa	1.5±0.4	1.0±0.7*	0.8±0.2	0.6±0.3*
Mucous neck	2.1±0.7	2.1±0.7	1.2±0.5	1.1±0.7
Basal mucosa	0.2±0.1	0.3±0.2	1.1±0.1	1.3±0.5

Data are expressed as mean±S.D. for 6—12 animals. *$p < 0.05$ compared with SO rats.

Localization of AM mRNA and AM-R mRNA

In SO rats, in situ hybridization with antisense probe demonstrated expression of AM mRNA in superficial mucosa, mucous neck cells and basal mucosa (*Fig. 2A*). AM-R mRNA had a similar distribution, although its expression in mucous neck cells, enterochromaffin like cells and especially the basal mucosa was stronger than that of AM (*Fig. 2B*). There was no marked expression of both AM mRNA and AM-R mRNA in the submucosa except in endothelia of submucosal vessels that had AM-R mRNA expression. In situ hybridization with sense probes (controls) did not show any mRNA, proving reaction specificity. In the superficial gastric mucosa (upper one-fourth of mucosal thickness) the intensities of both AM and AM-R were significantly reduced by 33% and 25%, respectively in PHT gastric mucosa compared with those of SO mucosa (*Table 1*).

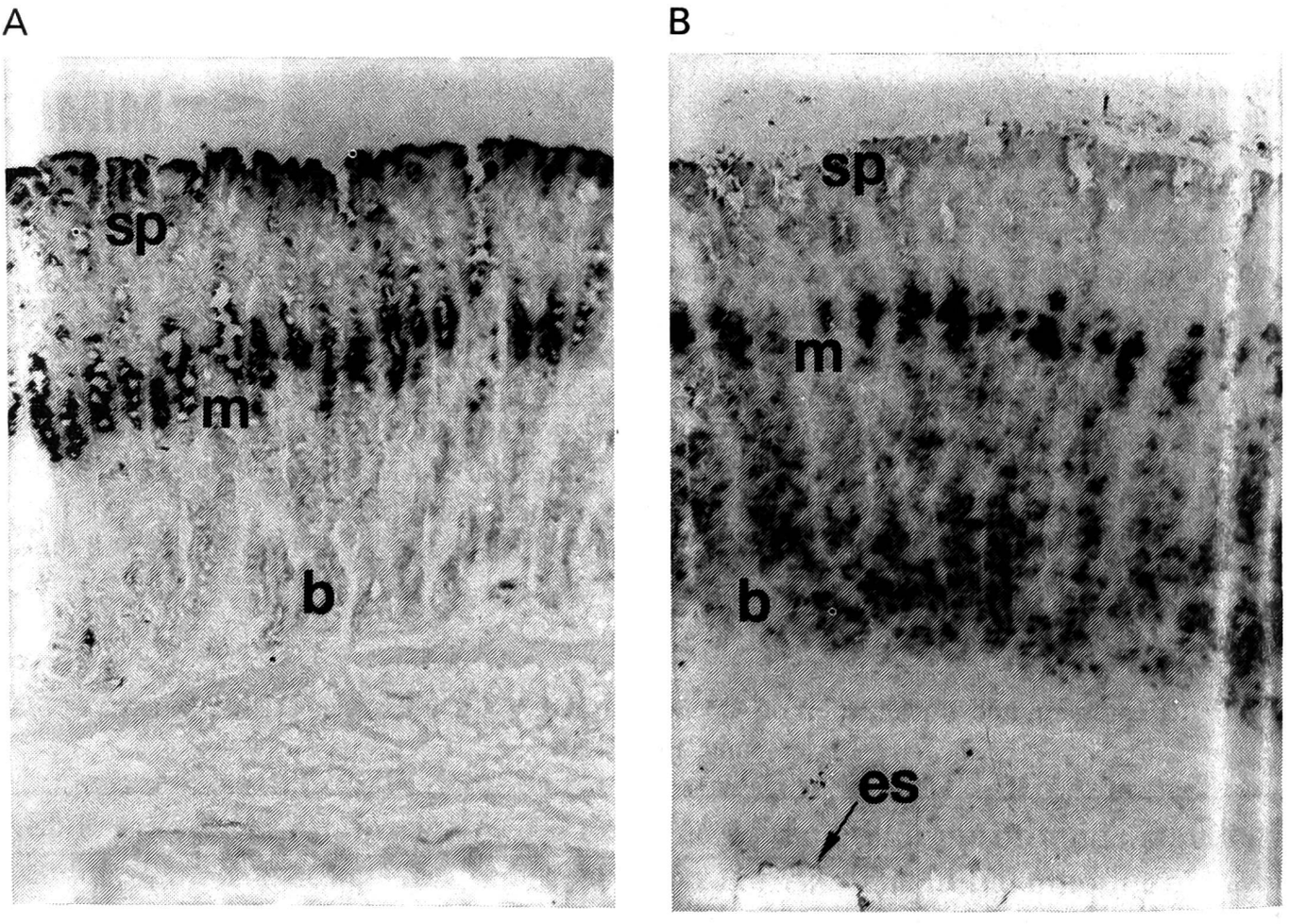

Fig. 2. Photomicrographs of in situ hybridization for adrenomedullin (AM) (A) and adrenomedullin receptor (AM-R) (B) in the gastric wall of sham operated (SO) rats × 100. sp: superficial mucosa, m: mucous neck area, b: basal mucosa, es: endothelium of submucosal vessels.

Expression of AM Protein

Western blot analysis demonstrated presence of AM protein (expressed as a 6 kDa band) in rat stomachs (*Fig. 3A*). There was no statistically significant difference between SO and PHT stomachs (*Fig. 3B*).

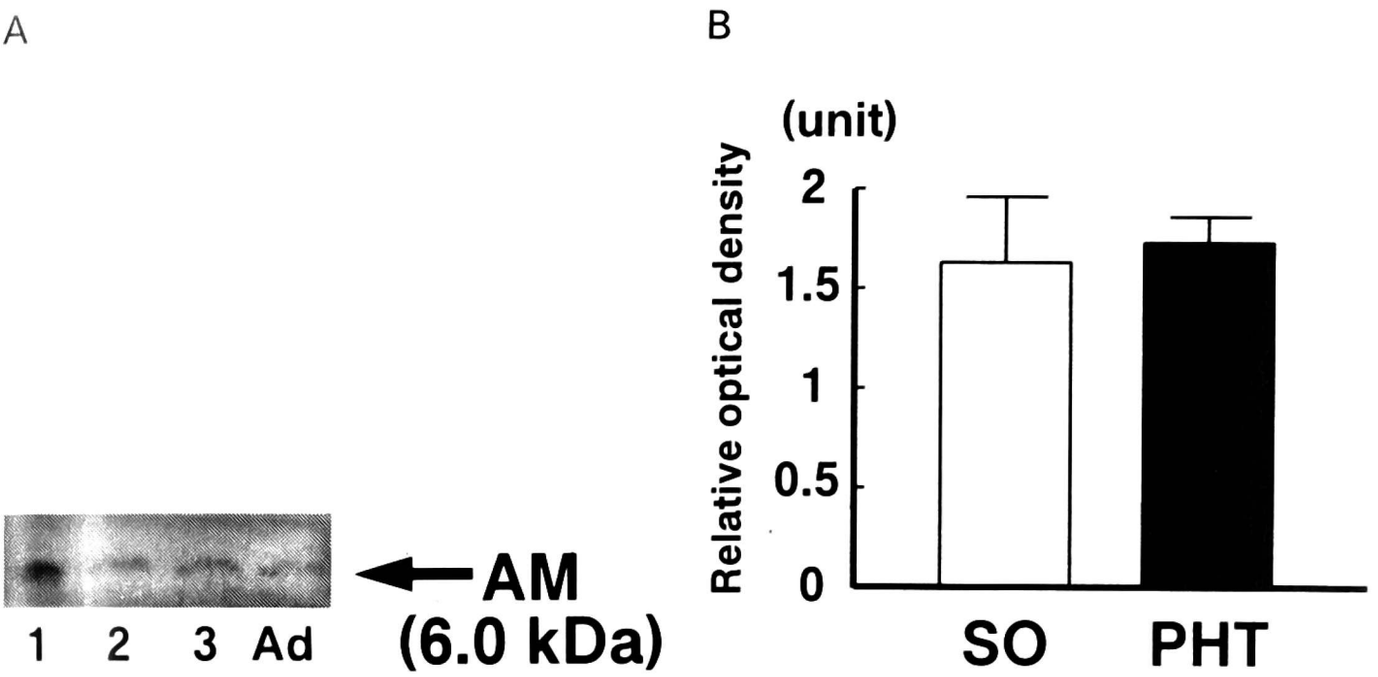

Fig. 3. (A) Western blot analysis of AM in rat gastric tissues. The AM protein is expressed as a 6.0 kDa band. Lane 1: SO rat, lane 2—3: PHT rats, Ad: Adrenal gland. (B) Quantitative analysis of AM protein expression in the gastric tissue using densitometric scanning of Western blots. The values are mean ± S.D. in densitometric units (n = 7 animals per group).

DISCUSSION

The expression and localization of AM and AM-R mRNAs in the gastrointestinal tract has not been extensively studied *in vivo*. Previous studies demonstrated that AM immunoreactive cells are present in the mucosa of stomach, small intestine and colon, and that they are mainly located at the base of the glandular epithelium (24). In addition, AM-like immunoreactivity has been found to be present in a subpopulation of enterochromaffin (serotonin-containing) like cells (25). Our present study demonstrated for the first time the expression and localization of AM mRNA, AM-R mRNA and AM protein not only in normal but also in PHT rat gastric mucosa. Although RNA blot analysis demonstrated less expression of AM in the rat stomach than in cultured endothelial cells or vascular smooth muscle cells (9), in the present study we examined AM mRNA expression using more sensitive and quantitative technique such as competitive RT/PCR. AM and AM-R mRNAs are expressed strongly in both SO and PHT stomachs in mucous neck cells and basal mucous cells, but there were no differences in total expression of AM and AM-R mRNAs between the two groups. While no differences were seen in total expression of AM and AM-R mRNAs between SO and PHT stomachs, our present study indicates relatively lower levels of AM and AM-R mRNA expression in superficial PHT gastric mucosa and increased levels in the basal mucosa.

We have previously demonstrated that gastric mucosal blood flow is significantly increased in PHT rats compared with SO rats. A recent clinical

study indicate that circulating levels of AM are increased in cirrhotic patients with ascites and correlate with hemodynamic abnormalities (19). Considering the vasodilatory effect of AM and AM-R, locally increased expression of AM and AM-R will produce increased blood flow in the basal mucosa. Conversely, reduced levels of AM and AM-R locally will result in decreased blood flow in superficial mucosa. Previous reports indicate that the gastric mucosal blood flow is increased in PHT rats (26—28). This increase in blood flow seems to occur predominantly at the mucosal base, since the capillary lumina in the middle portion of PHT gastric mucosa are narrowed (15, 16) and since the oxygenation of the luminal mucosal surface is significantly reduced (12). This hypooxygenation of the surface mucosa of PHT gastric mucosa previously demonstrated can be, in part, a result of a local reduction in the upper mucosa of AM and AM-R mRNAs and by inference corresponding proteins.

Mucosal resistance to injury ultimately depends upon a balance between defensive factors and aggressive factors present in the lumen. The microcirculation of the gastric mucosa is one of the most important defensive factors (8). Various vasoactive mediators (e.g. nitric oxide, prostacyclin) play important roles in mucosal defense (29). Our in situ hybridization study demonstrated that in the superficial mucosa of PHT stomachs both AM mRNA and AM-R mRNA are significantly less expressed than in that of SO stomachs. We have previously demonstrated that both acid-independent injury by ethanol and acid-dependent injury by aspirin of PHT gastric mucosa are significantly increased as compared with SO gastric mucosa (10, 11). Therefore, decreased expression of AM and AM-R mRNAs and by inference corresponding proteins in the superficial mucosa of PHT rats could be a cause of impairment of gastric mucosal defense. In normal rats, AM and AM-R may participate in regulation of the vascular tone in a paracrine/autocrine manner or play an important role in the mucosal microcirculation crucial for gastric mucosal defense.

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