Ribozyme compromise of adrenomedullin mRNA reveals a physiological role in the regulation of water intake

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Taylor, Meghan M., and Willis K. Samson. Ribozyme compromise of adrenomedullin mRNA reveals a physiological role in the regulation of water intake. Am J Physiol Regulatory Integrative Comp Physiol 282: R1739–R1745, 2002; 10.1152/ajpregu.00696.2001.—The adrenomedullin (AM) preprohormone is posttranslationally processed to result in two biologically active fragments, AM and proadrenomedullin NH2-terminal 20 peptide (PAMP). AM is thought to play a role in fluid and electrolyte balance by acting in brain to inhibit salt and water appetite and in the kidney to cause diuresis and natriuresis. We previously have shown that AM is necessary for the short-term regulation of salt intake. In this paper, we have designed a ribozyme, a catalytic RNA molecule, which specifically recognizes and cleaves the AM transcript. In cultured vascular smooth muscle cells, ribozyme treatment lowered AM mRNA and reduced peptide content. Intracerebroventricular administration of the ribozyme lowered hypothalamic AM content and led to an exaggerated drinking response in rats, demonstrating that endogenous, brain-derived AM is physiologically relevant and necessary for short-term control of water intake.

proadrenomedullin amino-terminal twenty peptide; fluid and electrolyte homeostasis; translation blockade

THE ADRENO M EDULLIN (AM) preprohormone is a 185-amino acid protein that is posttranslationally processed to result in two biologically active fragments, AM and proadrenomedullin NH2-terminal 20 peptide (PAMP). AM and PAMP are multifunctional peptides that are produced throughout the body. Both AM and PAMP are potent vasodilators, albeit via different mechanisms. AM stimulates the release of nitric oxide from endothelial cells (5), whereas PAMP acts presynaptically to inhibit the release of catecholamines from the adrenergic nerves innervating blood vessels (17). Elevated AM levels are found in many disease states (14), including essential hypertension, congestive heart failure, chronic renal failure, and sepsis.

The pharmacological actions of AM have been widely studied, leading to the hypothesis that AM is important in the regulation of fluid and electrolyte balance. We previously have shown that exogenously administered AM inhibits salt and water appetite (13, 16), actions that complement the peptide’s ability to cause diuresis and natriuresis (7, 23). The first pharmacological action of AM shown to be physiologically relevant was the ability of the peptide to inhibit salt intake in short-term peptide compromise studies (15).

We have been limited in our technical approach to the study of physiological relevance of AM and PAMP by the minimal available tools. The only available antagonist, AM 22–52, requires doses that exceed solubility in volumes employed in our central administration studies. We have employed successfully passive immunoneutralization in behavioral studies (16); however, specificity and duration of action are important concerns with this technique. A second method we have employed is blockade of peptide production with antisense oligonucleotides (15). If the appropriate antisense oligonucleotide can be designed and delivered to the cells producing the peptide, then the only limitations are the efficiency of entry into those cells and the limited duration of action.

Both methods described above suffer from their limited time of efficacy. A longer-term compromise of either peptide function or production would permit more extensive examination of the physiological role of AM in a given tissue and its relationship to its actions in other tissues. Unfortunately, embryonic compromise of AM production is lethal, pups dying at days 14.5–16.5 in utero (3). We, therefore, have taken the approach of designing a postdifferentiation, site-specific translation blockade of the peptide with a ribozyme targeted to the AM mRNA.

A ribozyme is a catalytically active RNA molecule that cleaves a complementary mRNA sequence, preventing protein production. One of the smallest known ribozymes is the hammerhead ribozyme, which is composed of ~30 nucleotides. It is a common sequence found in many plant viroids that undergo site-specific, self-catalyzed cleavage as part of their replication process (20). All hammerhead ribozymes have a common motif consisting of three base-paired helices connected by two invariant single-stranded regions forming the catalytic core. There are few conserved residues outside of the single-stranded catalytic core. Helix three...
contains the cleavage triplet, the site that is cut by the catalytic core. In nature the most commonly found cleavage triplet is GUC. However, mutagenesis studies have shown that any cleavage triplet with the sequence NUH is tolerated, where N is any nucleotide and H is A, U, or C (2). Hammerhead ribozymes catalyze the site-specific hydrolysis of the phosphodiester bond at the 3’-end of the cleavage triplet.

The native viroid ribozymes can be altered to create an intermolecular cleaving ribozyme by removing the terminating loop. This new hammerhead ribozyme then consists of two antisense arms surrounding the catalytic core. Because mRNA often folds into complex secondary structures, the accessibility of the target site to the annealing arms of the ribozyme is crucial to contemplate during ribozyme design. Arm lengths of seven to eight nucleotides are optimal to convey both specificity and access to most ribozymes (10). These shorter annealing arms also aid in turnover of the ribozyme, allowing each ribozyme molecule to cleave multiple target mRNAs (6).

We have designed a ribozyme that specifically recognizes and cleaves the preadrenomedullin transcript. This results in vitro in a reduction of AM protein content in cultured vascular smooth muscle cells (VSMCs). Intracerebroventricular injection of the ribozyme into rats also causes a reduction of AM protein levels, resulting in exaggerated water drinking after overnight water restriction or central administration of ANG II. This further supports the hypothesis that AM is an important contributor to the physiological maintenance of fluid and electrolyte homeostasis.

MATERIALS AND METHODS

Design of the ribozyme. We designed a ribozyme that was targeted to the region near the transcription initiation site because an antisense oligonucleotide from previous studies was successfully targeted to this region (15). Antisense arms of seven nucleotides each were chosen in accordance with the published data on optimized catalytic conditions (2). The remaining sequence of the catalytic core of the ribozyme was designed based on previously published sequences (2, 22). The sequence is as follows with the antisense arms underlined: 5’-GGGUGUGCUAGUCUCGGACAGCAGGACGAGCGC-3’ (Fig. 1). An RNA oligonucleotide that was methylated at the first and last six bases was obtained from Integrated DNA Technologies (Coralville, IA).

Northern analysis of ribozyme cleavage of AM mRNA. Rat VSMCs (a generous gift from Dr. Ellis Levin, Univ. of California, Irvine) were grown to confluence in six-well plates (12). Cells were transfected with 10 μg ribozyme oligonucleotide using 10 μl of a 2 mg/ml stock solution of lipofectamine (Invitrogen, Carlsbad, CA) in serum-free media. At various time points, total RNA was collected with TRIzol (Invitrogen). Samples were chloroform extracted, and RNA was precipitated in isopropanol. RNA was dissolved in diethyl pyrocarbonate-treated water and quantified by spectrophotometry. Samples were electrophoresed on a 2.5% formaldehyde agarose gel at 100 V for 75 min. The 18S ribosomal subunit was visualized with ethidium bromide staining to control for equal loading. The RNA was then transferred onto a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN) using a Turbo Blot device (Schleicher and Schuell, Keene, NH). A cDNA probe was made by RT-PCR using One-Step Superscript system (Invitrogen) and primers previously designed by Martinez et al. (11). The probe was digoxigenin-labeled using a DIG-High Prime Labeling Kit (Roche, Indianapolis, IN). After overnight hybridization with the probe, the blot was washed, and AM mRNA was detected by an alkaline phosphatase-labeled antibody against digoxigenin (Roche) and visualized with autoradiography film.

AM protein content in ribozyme-treated VSMCs. To show that relative levels of AM could be changed by ribozyme cleavage, VSMCs were grown to confluence in six-well plates. Cells were transfected with the AM ribozyme oligonucleotide (1–9 μg) or vehicle using 10 μl of a 2 mg/ml stock solution of
lipofectamine in serum-free media for 4 h. AM production was stimulated by adding lipopolysaccharide (LPS; 10 ng/ml, sterile type 026:B6, Sigma, St. Louis, MO) in DMEM plus 10% FBS and a 1:10,000 dilution of protease inhibitor cocktail (Sigma). The cells were incubated for 6, 12, 18, 24, 48, 72, or 96 h, and then the media and cells were collected after acidification with acetic acid (final concentration 0.5 M acetic acid). The samples were heated to 100°C for 10 min, sonicated, and then dried in a rotary evaporator. Once dry, the protein samples were resuspended in 125 μl PBS. A 10-μl aliquot of the resuspended protein was used to quantify the AM levels by RIA (Phoenix Pharmaceuticals, Belmont, CA). AM levels determined by RIA were normalized on the basis of total protein collected in each sample. We examined, by paired t-test, the effect of ribozyme treatment at defined intervals (treatment effect only) by comparing peptide content in LPS-treated cells that had also been exposed to the ribozyme (in lipofectamine) with that detected in LPS-treated cells exposed to lipofectamine alone. At one time point (24 h), we examined the concentration dependency of the ribozyme treatment effect by one-way ANOVA with a Bonferroni adjustment for multiple comparisons. An outcome with a probability of P < 0.05 was considered significant.

In vivo studies. Adult male rats (Sprague Dawley, 200–225 g, Harlan, Indianapolis, IN) were housed individually with free access to food and tap water (12:12-h light-dark cycle). An indwelling stainless steel cannula was placed into the right lateral cerebroventricle of the rat under tribromoethanol anesthesia (2.5% in saline, 1 ml/100 g body wt, Sigma) as previously described (15). Animals were allowed to recover to presurgery body weight (5% NaCl). Animals (n = 9 each) were then denied access to water for 18 h. Thereafter, water bottles were returned to the cages, and cumulative water intake was measured at 0.5-h intervals. A second group of animals (n = 14) with brain cannulas was administered 2 μg ribozyme or vehicle and denied access to water overnight. Animals were killed by rapid decapitation at 18 h. Hypothalami were removed and homogenized in 2 N acetic acid. Samples were dried in a rotary evaporator, and AM content was determined by RIA as described above. To test the dipsogenic response to ANG II, animals (n = 9 each) were administered 2 μg ribozyme intracerebroventricularly or saline vehicle. Six hours later the rats were administered 100 pmol of ANG II centrally. Cumulative water intake was measured every 15 min for 90 min. The effect of the AM ribozyme on thirst under normovolemic conditions was examined by administering 2 μg ribozyme or vehicle (n = 7 each) and measuring cumulative water intake hourly for 24 h. All in vivo data were analyzed by independent sample t-test comparing water intake in controls to that observed in ribozyme-treated rats at each time point.

**RESULTS**

**Northern analysis.** Ribozyme treatment led to a decrease in full-length AM RNA levels that was visible by 2 h after treatment (Fig. 2). AM RNA levels continued to decrease through the final 6-h sampling time point. **AM protein reduction in VSMCs.** To determine the efficacy of ribozyme treatment, VSMCs were treated with 3 μg ribozyme oligonucleotide or vehicle, and samples were collected at 6, 12, 18, 24, 48, 72, and 96 h after LPS stimulation. Ribozyme pretreatment of LPS-stimulated VSMCs led to an ~20% decrease in AM protein content (Fig. 3), evident by 12 h (P < 0.025). By 48 h after LPS treatment, ribozyme pretreatment led to a 50% reduction in AM content. This effect remained significant at 72 h (P < 0.005) but had resolved by 96 h. The concentration dependence of ribozyme was tested by treating VSMCs with 1, 3, or 9 μg of ribozyme oligonucleotide. Samples were collected after a 24-h incubation with LPS. Decreases in AM content were related to the amount of ribozyme in each treatment (Fig. 4). Both the 3- and 9-μg treatments were significantly different from control (P < 0.05); however, they were not significantly different from each other.

In vivo studies. Intracerebroventricular ribozyme treatment followed by 18 h of water restriction significantly reduced the amount of AM protein in rat hypothalami (0.98 ± 0.10 pg/mg total protein vs. 1.37 ± 0.13 pg/mg in controls, n = 14 each; P < 0.025). This reduction in AM was accompanied by a significantly greater drinking response than was observed in controls. Ribozyme-treated animals initiated drinking more rapidly than controls (Fig. 5) and continued to drink significantly more water than control animals for 150 min after the bottle was returned. No significant differences in cumulative water intake were observed 3 h after initiation of drinking or for the remaining interval. The dipsogenic response to central ANG II administration was also altered by ribozyme pretreatment. Ribozyme-treated animals displayed exaggerated water intake compared with controls (Fig. 6). These animals continued to drink for at least 15 min after the control animals were sated (P < 0.025). Under normovolemic conditions, ribozyme treatment had no effect on water intake (Fig. 7).

**DISCUSSION**

Due to the lethality of the AM knockout, we sought an alternative method to compromise AM and PAMP protein levels. The use of a ribozyme was appealing because it could be delivered in a site-specific manner, enabling us to study the central actions of AM and PAMP. One of the biggest benefits of ribozymes is that they can be inserted into the host genome by a virus, allowing for continual production and, thereby, sustained decreases in the target protein levels. Already groups have made ribozymes to treat dominant genetic diseases and found these ribozymes to be able to halt...
progression of the diseases in animal models (8, 9). Therefore, the use of a ribozyme to create a postdevelopmental, site-specific abrogation of AM and PAMP production is feasible and would enable the study of the preproadrenomedullin gene products in brain or other sites.

In vivo use of a ribozyme for protein ablation requires getting the ribozyme into cells. There are two methods of delivery. Exogenous delivery of a presynthesized ribozyme is the method we use in this paper. There are two main problems with this technique. The first problem is that once the ribozyme is taken up, it is quickly degraded. To overcome this problem, ribozymes are frequently chemically modified, as was ours. The second problem with exogenous delivery of the ribozyme is that, like immunoneutralization and antisense treatment, effects are relatively short lived. Our treatment lasted for only 72 h, based on the in vitro data.

The other method for delivering ribozymes is endogenous delivery as mentioned above. This is accomplished using viral vectors to insert a gene to produce the ribozyme. Expression cassettes can be designed to carry cell type-specific or conditional transcription initiation sites, as well as to include reporters. The big advantage of an endogenous ribozyme is that it can be continuously produced, allowing for the ablation of target protein production over a long period of time. This would enable the study of consequences of long-term protein loss to be undertaken. We have designed a viral construct carrying our ribozyme that will be used in future studies to examine the effects of long-term ablation of brain-derived AM and PAMP.

After designing the ribozyme, we had to ensure that the ribozyme would indeed cleave the AM gene transcript. For these studies, we obtained a methylated ribozyme oligonucleotide. Ribozyme cleavage and AM

![Fig. 3. Time-dependent ribozyme compromise of lipopolysaccharide (LPS)-stimulated AM production in cultured vascular smooth muscle cells (n = 6/group). *P < 0.05, **P < 0.025, ***P < 0.005 vs. control.](image)

![Fig. 4. Concentration-dependent ribozyme (Ribo) compromise of LPS-stimulated AM production in cultured vascular smooth muscle cells (n = 6/group). *P < 0.05 vs. control. **P < 0.01 vs. control.](image)

![Fig. 5. Effect of central administration of AM ribozyme on cumulative water intake in rats deprived of water overnight (18 h). *P < 0.05, **P < 0.01 vs. saline vehicle-injected control responses (n = 9 each).](image)

![Fig. 6. Effect of central administration of AM ribozyme on cumulative water intake in rats administered 100 pmol ANG II intracerebroventricularly. *P < 0.025, **P < 0.005 (n = 9 each).](image)
quantification were initially carried out in VSMCs. Transformed VSMCs are an ideal model to test the activity of the ribozyme construct because they produce large amounts of AM (13) and are relatively easy to maintain in culture (12). An added benefit is that substances that raise and lower levels of AM production are known for these cells (18).

Northern analysis was performed on RNA extracted from ribozyme-treated VSMCs to ensure the ribozyme was able to cleave the AM transcript. The decrease in full-length AM mRNA in ribozyme-treated VSMCs clearly demonstrated the ability of our ribozyme to cleave the transcript. A smaller band, the size of the cleaved product, was faintly visible in the ribozyme-treated samples. Because RNA fragments that cannot be translated are rapidly degraded, we are not surprised that we did not detect a great deal of cleaved transcript.

A change in RNA levels is not always reflected by a change in protein production, so we examined the AM protein content of ribozyme-treated VSMCs. LPS is a potent activator of AM production in VSMCs. In LPS-stimulated VSMCs, ribozyme pretreatment led to an ~20% decrease in AM protein content compared with controls (Fig. 3), evident by 12 h. Ribozyme-related decreases in AM protein were most significant 72 h after LPS treatment, where AM protein levels were reduced by 55% in the ribozyme-treated samples. At 96 h there was no difference in AM content between ribozyme-treated cells and control-treated cells probably due to destruction of the ribozyme. AM protein levels in both ribozyme-treated and control cells were also lower at 96 h, suggesting the stimulatory effects of the LPS had worn off. We also treated some samples with a ribozyme containing scrambled antisense arms (data not shown). There was no difference in AM protein between control vehicle-treated cells and scrambled ribozyme-treated cells. We did not examine PAMP levels in any of the samples. Because the two peptides are formed by the processing of a preprohormone, it was assumed that PAMP levels were also decreased by ribozyme treatment. The decrease in AM content was not only time dependent but was also related to the amount of ribozyme delivered (Fig. 4). At 24 h after LPS stimulation, a dose-related effect of ribozyme treatment could be seen. The threshold for significant effects was 2–3 μg of ribozyme. The small magnitude of change among the treatment groups may be due to inefficient cellular uptake or destruction of the ribozyme. Alternatively, it may reflect stored peptide produced before ribozyme treatment.

The ultimate goal of designing a ribozyme was to be able to employ it in vivo to ascertain the physiological role of endogenous AM and PAMP. Previously, our lab has shown that AM is physiologically important in the regulation of fluid and electrolyte intake. The effects of centrally administered AM on salt appetite in rats were examined using an isotonic, hypovolemic stimulus, polyethylene glycol (PEG). Rats treated with AM centrally during a PEG challenge ingested significantly less salt compared with controls, without altering total fluid intake (16), suggesting that AM might be a “satiation” cue for salt ingestion. On the other hand, blockade of peptide action using passive immunoneutralization (16) or abrogation of peptide production using an antisense oligonucleotide (15) resulted in exaggerated salt intake without altering total water intake to the same isotonic hypovolemic stimulus. Thus endogenous AM plays a physiologically relevant role in the central nervous system control of electrolyte balance.

Additionally, our lab has examined the effects of AM on fluid intake (13). Intracerebroventricular administration of AM inhibited water intake to a mixed hypovolemic/hyperosmotic challenge (water deprivation) or a hyperosmotic challenge alone (intraperitoneal injection of hypertonic NaCl). AM was also able to diminish ANG II-stimulated water intake in a dose-dependent manner. Thus we hypothesized that compromise of AM production by the ribozyme would lead to increased water intake after a period of water restriction or after central ANG II administration. Indeed, this was the case.

Ribozyme-treated rats had significantly lower hypothalamic levels of AM than did control rats after 18 h of water deprivation. Again, residual peptide levels may reflect preformed AM or populations of AM neurons that were not accessed by the ribozyme when injected centrally. A second group of ribozyme-treated rats was also denied access to water overnight. Once water was returned, total intake was measured. The ribozyme-treated rats exhibited an exaggerated drinking response (Fig. 5). They went immediately to the water bottle and consumed significantly more water than controls for the first 150 min after water was returned. No significant differences in cumulative water intake were observed 3 h after initiation of drinking or for the remaining interval. This may reflect the transient nature of the effect of single bolus administration of the ribozyme or the fact that other neural factors important in the control of water intake compensated for the
absence of endogenous AM and PAMP. Another possible explanation, however, is that by 150 min, the ribozyme-treated rats had consumed enough water to negate the initial dehydration stimulus.

ANG II is a known dipsogenic factor. When 2 μg ribozyme were administered intracerebroventricularly 6 h before administration of 100 pmol of ANG II also intracerebroventricularly, exaggerated water drinking was observed compared with vehicle-treated controls (Fig. 6, n = 9). Total water intake measured at 24 h after ANG II treatment did not differ between treatment groups. Thus reduction of endogenous, brain-derived AM removes a “brake” on thirst during a mixed volume and osmotic stimulus (dehydration) or a purely pharmacological stimulus (ANG II). Although in the studies reported here water drinking in response to ANG II was initiated 6 h after ribozyme administration, it will be important in future studies to determine the temporal length of efficacy of the translation compromise. It could be that, because the ribozyme was effective for up to 72 h in vitro (Fig. 3), the effect we observed after 6 h in the ANG II-induced drinking paradigm is maintained for longer periods. If that were the case, then an even broader window of experimentation would be available with this approach. It is clear from our overnight dehydration paradigm (Fig. 5) that the ribozyme compromise was effective 18 h after administration of the oligonucleotides.

We chose two established, yet unique, paradigms for the stimulation of water drinking behavior. ANG II administration is a purely pharmacological stimulus for water drinking. Water restriction, on the other hand, provides multiple stimuli for water drinking behavior. The resulting hypovolemia activates renin release with subsequent generation of the dipsogenic peptide ANG II. Hypovolemia also results in changes in afferent input from the arterial baroreceptors that can contribute to the stimulus for water intake. In addition, the increased plasma osmolality is sensed directly by cellular elements in the forebrain. Thus multiple neuronal inputs converge on the networks controlling thirst, and numerous neural factors (transmitters, neuropeptides) are recruited. Ribozyme-treated animals consumed significantly more water already at 15 min after water bottle reintroduction in the water restriction model. This would suggest that the inhibitory effects of endogenous AM are exerted on multiple thirst-stimulating pathways or at their convergence. Because the stimuli were well in place at the time of bottle reintroduction, it is not surprising that the result of the absence of an inhibitory factor was observed early in the drinking response. In the case of the ANG II-induced drinking, significant differences in water intake were not observed between the two groups until the 30-min time point. It is possible that the drinking observed at the 5- and 15-min time points reflects increased locomotor activity attendant to the intracerebroventricular injections, which required the brief approach of the investigator to the animals. However, it is also possible that the inhibitory effects of endogenous AM (compromised in this experimental approach) are only expressed after the initial activation of the ANG II receptor. In the water restriction model, the activation of the ANG II receptor would have occurred well before the reintroduction of the water bottle.

Ribozyme treatment had no effect on water drinking in normally hydrated rats (Fig. 7). This finding is not surprising as the actions of many peptides cannot be seen under basal conditions but become obvious under stimulated conditions. Indeed, central administration of AM does not alter water drinking behavior in satiated rats, but instead can only be observed to alter water drinking when the behavior is stimulated. These data suggest that the AM gene products control short-term fluid intake and thus regulate cardiovascular function at least in part by maintaining normal plasma volume and osmolality.

In summary, we have designed a ribozyme that specifically recognizes and cleaves the preproadrenomedullin transcript. This results in a reduction of AM mRNA and protein content in cultured VSMCs. Intracerebroventricular administration of the ribozyme into rats also causes a reduction of AM protein levels in hypothalamus, resulting in exaggerated water drinking after overnight water restriction or in response to central ANG II administration. These data support our hypothesis that AM is an important contributor to the physiological maintenance of fluid and electrolyte homeostasis.

**Perspectives**

Our initial studies identified a pharmacological effect of exogenous AM on water drinking (13). This suggested that endogenous AM plays a physiologically relevant role in the control of water drinking, as we have demonstrated for another ingestive behavior, salt appetite (15, 16). While passive immunoneutralization and antisense oligonucleotide treatment provide a short-term blockade of peptide function or production, we sought to develop a longer-term compromise of the action of endogenous, brain-derived AM. We developed a ribozyme that can interrupt the translation of the AM gene and reduce cellular peptide content. With this approach, we have demonstrated that, in the absence of endogenous AM, water drinking in response to physiological and pharmacological stimuli is significantly exaggerated. It will be important to extend this methodology, more permanently compromising endogenous peptide production so that the role of AM in the long-term regulation of fluid and electrolyte homeostasis and cardiovascular function can be examined (21). Employment of viral transfection strategies will allow for continuous expression of our ribozyme in vivo, facilitating not only the examination of those long-term effects but also the identification of possible compensatory mechanisms that may be recruited in the absence of this peptide.

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REFERENCES