Long-Lasting Cardiovascular Effects of Liposome-Entrapped Angiotensin-(1-7) at the Rostral Ventrolateral Medulla

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Abstract—The aim of this work was to evaluate the potential of liposomes as a tool for the sustained release of the short half-life peptides of the renin-angiotensin system in a specific site of the brain. Angiotensin (Ang)-(1-7) was selected for this study because of its known cardiovascular effects at the level of the rostral ventrolateral medulla (RVLM) and because of the considerable interests in elucidating its physiopathological role as a neuromodulator. Ang-(1-7)–containing liposomes (LAng) were microinjected unilaterally in the RVLM of Wistar rats, and the effects on blood pressure (MAP) and heart rate were evaluated by telemetry. Empty liposomes (Lemp) were used as control. LAng elicited a significant pressor effect during daytime and bradycardia during nighttime that lasted for 5 and 3 days, respectively. These cardiovascular effects resulted in a significant attenuation of the circadian variations of MAP and heart rate. In the case of MAP, a significant inversion of the circadian rhythm was observed on day 2 after LAng microinjection. None of these effects were observed following microinjection of Lemp. Using this novel technique, it was possible to establish, in chronic conditions, the pressor effect of Ang-(1-7) at the RVLM. Moreover, our data unmasks a new physiological role for Ang-(1-7) at the level of the RVLM: modulation of the circadian rhythms of MAP and heart rate. (Hypertension. 2001;38:1266-1271.)

Key Words: liposomes ■ angiotensin-(1-7) ■ cardiovascular effects ■ rostral ventrolateral medulla ■ Blood pressure ■ heart rate

Several recent studies indicate that peptides of the renin-angiotensin system (RAS) may act as important neuromodulators, especially in the brain medullary areas related to the tonic and reflex control of arterial pressure.1 Experimental evidence was obtained following site-specific microinjections of these peptides; however, because of their very short in vivo half-life, only their acute cardiovascular effects could be observed.2–6 Therefore, information is still lacking on the long-term effects of these peptides, which should allow a better understanding of their physiopathological role. To address this question, injectable microreservoirs, which could act as sustained release systems in specific sites of the brain, have to be developed. Liposomes appear to be good candidates because of their biocompatibility and their recognized potential for the encapsulation and delivery of polypeptides in vivo.7 Moreover, liposome encapsulation was previously shown to produce a marked prolongation of the peripheral effect of some vasoactive peptides, including angiotensin (Ang) II,8 vasopressin,9 and vasoactive intestinal peptide.10

In this work, we evaluated the potential of liposomes for the sustained release of RAS peptides in a specific site of the brain. Ang-(1-7) was selected for this study because of its known cardiovascular effects at the level of the rostral ventrolateral medulla (RVLM)2–5 and because of the considerable interests in its long-term physiopharmacological effects.11 More specifically, liposome-encapsulated Ang-(1-7) was microinjected unilaterally in the rat RVLM, and the effects on blood pressure and heart rate (HR) were evaluated by telemetry.

Methods

Materials

Ang-(1-7) was obtained from Bachem. L-α-distearoyl-phosphatidylcholine (DSPC) and cholesterol (CHOL) were purchased from Sigma Co. Distearoyl-phosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG2000) was obtained from Avanti Polar Lipids Inc. 1,1,3,3-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Molecular Probe. All other reagents were of reagent grade and used without further purification.

Animals

Wistar rats (males weighing 250±10 g), bred at the animal facility of the Biological Sciences Institute (CEBIO, UFMG, Belo Horizonte, Brazil).
Preparation of Liposomes
Liposomes with incorporated Ang-(1-7) were prepared according to the dehydration rehydration vesicles (DRV) method, using a lipid composition of DSPC, CHOL, and DSPE-PEG2000 at a molar ratio of 5:4:0.3. Small unilamellar vesicles (SUVs) were prepared by sonication of the lipid mixture in distilled water at 55 °C, using a 3-mm probe-sonicator (Misonix, ultrasonic liquid processor). Residual large vesicles and contaminating metal from the probe were eliminated by centrifugation at 10000 g for 10 minutes; 1 mL of SUVs (lipid concentration, 90 mmol/L) was mixed with 1 mL of a 0.015 mol/L NaCl aqueous solution containing 1.4 g/L Ang-(1-7). The mixture was immediately frozen and then dried overnight. Rehydration of the dried powder was performed at 55 °C as follows: 0.2 mL of distilled water was added, and the mixture was vortexed and incubated 30 minutes at 55 °C; 0.2 mL of PBS (10 mmol/L phosphate, 0.15 mol/L NaCl) was similarly added, and the mixture was vortexed before the addition of 0.8 mL of PBS and incubation for 30 minutes at 55 °C. The resulting lipidosome suspension was extruded 10 times through 2 stacked polycarbonate filters of 200-nm pore size at 65 °C and pressure of 200 to 500 psi using an Extruder (Lipex Biomed membranes).13 The liposomes were then separated from nonencapsulated peptide by dialysis against PBS and, finally, sterilized by filtration through sterile 0.22-μm filters. Empty liposomes were obtained, using the same procedure but omitting the peptide in the preparation.

The size of the liposomes in the resulting preparations was analyzed by quasi-elastic laser light scattering (QELS, Model BI-900). The average vesicle diameter was found to be 0.19 μm for both empty and Ang-(1-7)-containing liposomes.

Dil-labeled liposomes were prepared essentially according to the method of Claassen. Briefly, Dil dissolved in ethanol at 2 mg/mL was added to the empty liposome suspension at a final concentration of 10 μg/mL and incubated for 60 minutes at 60 °C. The preparation of labeled liposomes was then dialyzed against PBS for 24 hours at 25 °C to eliminate residual ethanol.

Incorporation Efficiency of Ang-(1-7) in Liposomes
To determine the amount of Ang-(1-7) encapsulated in the final liposome preparation, a 50 μL aliquot was removed and added to 1950 μL of methanol to disrupt the liposomes. The amount of encapsulated Ang-(1-7) was then determined using Ang-(1-7) intrinsic fluorescence (maximum absorption, 278 nm; maximum emission, 315 nm). A weight ratio of encapsulated peptide to lipid of 0.03 (w/w) was found. Ang-(1-7) was also quantified by radioimmunoassay, as described below. Both methods gave similar values of encapsulation efficiency.

In Vitro Release of Liposome-Encapsulated Peptide
The ability of liposomes to retain encapsulated Ang-(1-7) was evaluated in PBS at 37 °C. After different times of incubation (24, 72, and 300 hours), aliquots were removed and submitted to ultrafiltration through Microcon YM-50 (50,000 NMWL, Millipore Corp). The amount of Ang-(1-7) recovered in the filtrate was then analyzed by radioimmunoassay, as described below.

Microinjection of Liposome Preparations
At the end of day 0 (between 2:00 PM and 6:00 PM), liposome-encapsulated Ang-(1-7) was unilaterally microinjected (35 ng/200 nL) into the RVLM with an injection needle (30 G) that was slowly inserted in the brain tissue through the dorsal surface, using the same stereotaxis coordinates (1.8 mm anterior, 1.8 lateral to the obex, and just above the pia-mater) as those used previously for acute experiments in awake rats.4 Empty liposomes or Dil-labeled liposomes were microinjected similarly at the same lipid dose. At the end of each experiment, the brain was then carefully removed and the position of the tip of the microinjection needle was macroscopically and microscopically examined to further confirm the location of the injection site in the RVLM.

Measurements of Arterial Blood Pressure and Heart Rate
About 2 weeks before microinjection, an arterial catheter (model PA11-C40, Data Science) was implanted under trichloroethanol anesthesia (250 mg/Kg) into rat abdominal aorta. Arterial blood pressure and heart rate were registered by telemetry for 10 seconds every 10 minutes, before microinjection and for up to 12 days after microinjection in undisturbed freely moving rats.

Localization Study of Liposomes
At t=0, rats were microinjected with 200 nL of dil-labeled liposomes into the RVLM. Animals were euthanized 24 hours and 7 days after microinjection. Frozen brains were cut into 16-μm sections. Dil fluorescence was observed as red with an Axioplan Zeiss fluorescence microscope, with BP 545 excitation filter and O 570 emission filter.

Radioimmunoassay for Ang-(1-7)
Radioimmunoassay for Ang-(1-7) was performed as described before.15 Before radioimmunoassay, the samples were purified using C18 Bond Elute cartridges.

Statistical Analysis
Data for blood pressure and HR were analyzed by ANOVA for repeated measures (Prism 3.0, GraphPad Software Incorporated). Acrophase, which represents the time of the daily peak of the circadian rhythm, was determined using the software DQ-fit and CV-sort.16 Comparison of acrophases were made using Watson’s F-tests (Software Oriana, Version 1.06, Kovach Computing Services).

Results
Characterization of the Preparation of Liposome-Encapsulated Ang-(1-7)
The encapsulation of Ang-(1-7) in liposomes was achieved with an efficiency of 12% and a ratio of encapsulated peptide to lipid of 0.03 (w/w). The resulting liposome suspension showed an average vesicle diameter of 190 nm. The fact that peptide quantification by radioimmunoassay and by intrinsic fluorescence analysis gave comparable values indicated that Ang-(1-7) did not suffer any significant alteration in the process of liposome preparation. The ability of liposomes to retain encapsulated peptide was very high, as >98% of originally encapsulated peptide was found to be retained following a 5-day incubation at 37°C in phosphate-buffer saline (data not shown).

Effects of Liposome-Encapsulated Ang-(1-7) on Arterial Blood Pressure and HR
Two groups of rats were microinjected unilaterally into the RVLM with 200 nL of liposome suspension containing either 35 ng of encapsulated Ang-(1-7) (LAng) or encapsulated PBS (Lemp). Arterial blood pressure and heart rate were registered by telemetry, for 4 days before microinjection and for up to 10 days after. From telemetry data, daytime and nighttime mean arterial blood pressure (MAP) and mean HR could be determined on each day for each animal.

Figure 1 displays the values of MAP and HR before and after microinjection. Microinjection of LAng elicited a sig-
significant pressor effect during the daytime that was maintained for 5 days. The highest blood pressure was obtained on day 3 (114±4 mm Hg), which differed significantly from that registered on day 0 (100±3 mm Hg). As expected, Lemp did not produce any significant pressor effect (94±5 mm Hg on day 3 versus 90±5 mm Hg on day 0). Moreover, MAP was found to be significantly higher in the LAng group than in the Lemp group on days 1, 2, and 3. Nighttime MAP, in contrast to daytime MAP, was not significantly affected by the microinjection of LAng, even though nighttime MAP in the LAng group differed significantly from that in Lemp group, on days 1 and 4.

Microinjection of LAng produced a significant decrease of HR during nighttime, which was maintained for at least 3 days (Figure 1). The peak of bradycardia occurred on day 2 with a nighttime value (319±9 bpm) that differed significantly from that obtained on day 0 (360±6 bpm). Such an effect was not observed during daytime or following microinjection of Lemp.

The effect of LAng on daytime MAP and its lack of effect on nighttime resulted in the alteration of the normal circadian fluctuations of MAP. Figure 2 shows that the usual difference between the daytime and the nighttime MAP was altered for the 5 days that followed the microinjection of LAng. Strikingly, MAP on day 2 was found to be significantly higher during daytime than during nighttime (paired t test, \( P<0.05 \)). This apparent inversion of the circadian rhythm of MAP was confirmed by the mean acrophase determined on day 2 in LAng group (1:07 PM), which differed significantly from the mean acrophases found on day 0 (11:53 PM) and on day 2 (10:42 PM) in Lemp (Watson’s F tests, \( P<0.05 \)).

Similarly, the effect of LAng on nighttime HR and its lack of effect on daytime HR resulted in the alteration of the normal circadian variations of HR. As illustrated in Figure 2, the usual difference between the daytime and the nighttime HR was altered for the first 2 days following microinjection of LAng, and it returned progressively to original levels in the following days. In contrast, Lemp did not show such an effect. Strikingly, the difference between the daytime and the nighttime HR on day 2 was found to be lower in the LAng group than in the Lemp group (unpaired t test, \( P<0.05 \)). On day 2, only 1 out of 5 animals showed an HR acrophase shifted to daytime (data not shown). Thus, the inversion of the circadian rhythm observed in the case of MAP could not be confirmed in the case of HR.

Localization of Liposomes

The aim of these experiments was to evaluate the localization and permanence of liposomes after microinjection and to confirm that most of the injected liposomes remained at the site of injection in the course of the experiment and do not migrate to areas other than RVLM. Empty liposomes were labeled with the lipophilic fluorescent dye, diI, and their localization was visualized under a fluorescent microscope in serial sections of the microinjection area. Figure 3 displays the fluorescence microscope images of the most fluorescent sections obtained 24 hours and 7 days after microinjection. The red fluorescent region was recognized as a part of the RVLM, mostly the paragigantocellular nucleus. This data
strongly suggests that liposomes remained concentrated in the RVLM and were removed very slowly from the microinjection site.

**Discussion**

We have previously shown that microinjection of Ang-(1-7) into the RVLM of anesthetized or awake rats produced an increase in MAP, whereas microinjection of its selective antagonist, A-779, induced a significant fall in MAP. These findings were the first to support the hypothesis that Ang-(1-7) or an Ang-(1-7)-like peptide would act as an endogenous neuromediator at this specific site and be involved in the central control of arterial blood pressure. In these experiments, microinjection of 25 to 50 ng of Ang-(1-7) produced an MAP increase of 15 mm Hg for minutes. The short duration of this effect was attributed to the rapid in vivo metabolism of the peptide. A major limitation of these studies was that they could not give information on the long-term cardiovascular effects of the peptide, which would allow for a better understanding of its physiopathological role.

The methodology developed in the present work represents a significant advance over conventional techniques. Liposomes are expected to protect encapsulated peptide from enzymatic degradation and to release it slowly at the site of injection. Such a behavior was previously reported in the case of liposomes containing Ang II, vasopressin, and vasoactive intestinal peptide that were administered by the subcutaneous or intravenous routes. In our study, the lipid composition and size of the liposomes were selected on the basis of experience previously acquired with liposomes administered subcutaneously, so as to obtain the most efficient sustained release system. First, liposome membrane was made from the high-phase transition temperature phospholipid, DSPC, to obtain a membrane of very low permeability. The expected high level of retention of our liposomes was clearly confirmed in our study. Secondly, a lipid derivative of poly(ethylene glycol) (DSPE-PEG2000) was introduced within liposome membrane, which has proven to oppose macrophage uptake. Finally, the size of liposomes was chosen such as to be large enough (diameter >120 nm) to prevent their migration from the injection site to the regional lymph nodes. The long permanence of liposomes at the site of injection was also confirmed in our study. Based on the in vitro stability of our liposome preparation at 37°C, one would expect a much more prolonged in vivo response than that actually observed. At least 2 different mechanisms may account for this discrepancy. First, even if coating the liposome surface with poly(ethylene glycol) is expected to oppose phagocytosis, this surface modification may affect the rate rather than the extent of cell uptake, as previously suggested. Therefore, cell capture and degradation of liposomes could be the main in vivo mechanism of peptide release. Second, some physiological regulatory mechanisms of blood pressure may also enter into action, a few days after microinjection, in response to the daytime increase in MAP.

The main cardiovascular effects that resulted from the unilateral microinjection of LAng into the RVLM were a small but significant pressor effect during daytime and a decrease of HR at night, that lasted for at least 3 days.

![Figure 2. Comparison between daytime (open bars) and nighttime (solid bars) MAP or HR, before and after microinjection of LAng (left) or Lemp (right) into the rat RVLM. Values are mean±SEM (n=5 per group). Liposome preparations were microinjected at the end of day 0. *P<0.05, comparisons between daytime and nighttime values (paired t test).]
Consequently, the slow release of Ang-(1-7) from the liposomes was found to alter the normal circadian variation of MAP and HR for at least 2 days. These effects could not be attributed to a mechanical stimulation, because empty liposomes did not affect significantly MAP or HR. Remarkably, these effects were achieved with a unique unilateral administration of a low dose of Ang-(1-7) (<50 pmol). The functional relevance of these Ang-(1-7) actions is reinforced by the fact that the sustained cardiovascular effects were elicited in normal undisturbed freely-moving rats and surpassed, at least partially, buffering cardiovascular reflexes.

Our present study brings important information about the modulatory activity of Ang-(1-7) at the RVLM, uncovering a circadian cycle–dependent influence of the heptapeptide on RVLM activity and/or responsiveness. Concerning the mechanisms underlying the selective daytime increase in blood pressure produced by LAng, some potential explanations can be raised, such as a direct excitatory effect of Ang-(1-7) on sympathetic premotor neurons increasing sympathetic activity or an increase in RVLM responsiveness to other excitatory inputs. Nevertheless, we cannot completely exclude the possibility that the MAP and HR changes could be owing to the effect of Ang-(1-7) on cells other than the sympathetic premotor neurons in the RVLM. The fact that Ang-(1-7) pressor activity was observed specifically during the daytime strongly suggests that the long-lasting effect of LAng did not result from a nonspecific increase of sympathetic-related neuronal activity. One may argue, however, that the decrease in HR in the dark phase may have masked a pressor action of Ang-(1-7) during the night period. Although we cannot completely discard this possibility, the differences in MAP and HR changes, from the point of view of their duration and acrophase, argues against this explanation. Circadian variations of the buffering capacity of cardiovascular reflexes, which may be influenced by the level of arousal, should be explored in future studies. However, it should be pointed out that at least for the baroreflex sensitivity, this possibility is unlikely because this reflex is decreased in the awake state compared with the sleep period.

The differential effect of Ang-(1-7) on MAP and HR is consistent with previous studies suggesting that the diurnal control of MAP is different from that of HR. In hypertensive transgenic rats harboring the mouse REN2 gene [TGR(mREN2)27] (TGR), MAP was reported to be higher in the light-phase than in the dark-phase, whereas the circadian rhythm of HR was not affected. Moreover, Ang-(1-7), acting at the RVLM, was shown to contribute to the hypertensive levels of MAP in these animals. Therefore, our current observation of the inversion of the circadian rhythm of MAP in response to the infusion of Ang-(1-7) opens the possibility that the heptapeptide might contribute to the changes in MAP fluctuations in TGR rats and in other RAS-dependent models of hypertension.

The changes in HR uncovered a significant influence of Ang-(1-7) in neuronal elements conveying circadian fluctuations of vagal and/or sympathetic drive to the heart. Our data are in accordance with previous studies performed in the daytime period showing inconsistent changes in HR after microinjection of Ang-(1-7) into the RVLM. The fact that HR changes occurred preferentially at night could not be attributed to a decreased locomotor activity (data not shown). Ang-(1-7) may have prevented the normal increase of HR on nighttime possibly by enhancing the vagal activity to the heart. As another possible explanation, Ang-(1-7) may have increased the responsiveness of the RVLM to neuronal discharges from other brain systems, such as the ventromedial nucleus of the hypothalamus (VMH). Neuronal inputs from the VMH decrease the neuronal activity of cardiovascular related neurons at the RVLM. However, these discharges, which occur every 15 to 30 minutes in the light phase, are scarce in the dark phase. Future studies are obviously needed to clarify the exact mechanism of the HR changes.

Two significant advances can be highlighted from our study. From the methodological point of view, it is the first time that the potential of liposomes as a sustained release system in a specific site of the brain is demonstrated.
the elucidation of the physiological role of RAS peptides in the brain. Indeed, the experiments described here do not represent exhaustive study of the potential of liposomes. Future studies are necessary to investigate the effect of particle size, lipid composition and ratio of encapsulated peptide to lipid on the release of liposome-encapsulated peptide from the microinjection site. Manipulations of liposome characteristics may then present another possibility to modulate the physiological response to encapsulated peptide. From the physiological point of view, this study established, in chronic conditions, the pressor effect of Ang-(1-7) at the level of the RVLM. Remarkably, it unmasked a new physiological role for Ang-(1-7) at the level of the RVLM: modulation of the circadian rhythms of blood pressure and heart rate.

Acknowledgments

We are indebted to Dr Conceição R.S. Machado and Dr Elizabeth R.S. Camargo (Departamento de Morfologia, ICB, UFMG) for their assistance with the fluorescence microscopy experiments. We also thank Dr Pedro Licinio (Departamento de Fisica, ICEX, UFMG) for the QELS determination of the size of liposomes. We thank José Roberto Silva and Soraia Silvéria Silva for their technical support and would like to acknowledge the Brazilian agencies, FINEP (PRONEX), CNPq, and CAPES for financial support.

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Hypertension. 2001;38:1266-1271
doi: 10.1161/hy1201.096056

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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