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Site-specific microinjection of liposomes into the brain for local infusion of a short-lived peptide

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Abstract

The short-lived peptide, angiotensin-(1-7) (Ang-(1-7)), was encapsulated in different liposome preparations, in order to evaluate the influence of membrane fluidity, membrane surface, liposome size and dose of peptide on the cardiovascular effects of the encapsulated peptide at a specific site of the brain. These preparations were microinjected unilaterally into the rostral ventrolateral medulla (RVLM) of Wistar rats, and mean arterial blood pressure (MAP) and heart rate (HR) were registered by telemetry. Pegylated, rigid and calibrated (200 nm) liposomes, containing 50 ng of Ang-(1-7), elicited a significant increase of MAP for at least 7 days, in contrast to empty liposomes or non-pegylated liposomes. When a two-fold higher peptide dose was employed or when pegylated liposomes were used in the fluid state or uncalibrated, less pronounced pressor effects were observed. These data show that the cardiovascular responses to the microinjection of Ang-(1-7)-containing liposomes into the RVLM can be modulated through the manipulation of liposome characteristics. These results can be explained by the influence of liposome characteristics on the flux of peptide release. It is expected that this new method will encounter numerous applications in the study of the chronic actions of short-lived bioactive peptides in specific sites of the brain.

Keywords: Liposomes; Pegylation; Angiotensin-(1-7); Rostral ventrolateral medulla; Renin–angiotensin system

1. Introduction

A major obstacle to the study of the physiology of short-lived peptides is the lack of appropriate methodology for assessing their chronic actions. This is particularly true when these actions have to be evaluated in a specific site of the brain and in freely moving awake animals.

Several recent studies indicated that angiotensin-(1-7) (Ang-(1-7)), an endogenous short-lived peptide of the Renin–Angiotensin System, acts as an important neuromodulator at the rostral ventrolateral medulla (RVLM), a brain area related to the tonic and reflex control of arterial blood pressure [1]. Microin-

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jection of Ang-(1-7) into the RVLM of normotensive rats resulted in a significant increase of arterial pressure [2]. This effect was found to be mediated by a specific surface receptor, presumably the G protein-coupled receptor Mas [3] in sympathetic premotor neurons, as microinjection of the Ang-(1-7) receptor Mas antagonist (A-779) induced a significant fall of blood pressure and heart rate [3]. In these studies, 25 to 50 ng of Ang-(1-7) produced an increase of 15 mm Hg for about 10 min. The short duration of this effect was attributed to the rapid in vivo metabolism of the peptide, essentially through inactivation by the ectoenzyme, Angiotensin-converting enzyme [4].

To get insight into the chronic cardiovascular actions of this peptide at the RVLM, a method was recently introduced that combines and takes advantage of three different techniques: liposome encapsulation, site-specific microinjection and telemetry. Ideally, liposomes may be designed to remain located at the injection site for a long period of time, where they protect encapsulated peptide from rapid degradation and act as a sustained release system. Secondly, microinjection allows the administration of bioactive substances in some specific sites of the brain with minimal side effects. Finally, using telemetry, it is possible to register physiological parameters and their circadian variations in undisturbed freely moving animals for days and even months. In a previous work, it has been shown that microinjection of liposome-containing Ang-(1-7) into the RVLM elicited a prolonged pressor effect for several days [5], in contrast to the free peptide that increased blood pressure for only a few minutes [2]. This long-lasting action also led to unmask a new physiological role for Ang-(1-7): its modulation of the circadian rhythms of mean arterial blood pressure [5]. However, in order to fully evaluate the potential of this new method and, more specifically, the possibility of manipulating the peptide actions through the control of its release rate from liposomes, the influence of liposome characteristics, such as vesicle size, membrane surface, membrane fluidity and amount of encapsulated peptide, still has to be determined. The present study reports the influence of liposome characteristics on the cardiovascular actions of liposome-encapsulated Ang-(1-7) at the RVLM.

2. Materials and methods

2.1. Materials

L-α-distearoylphosphatidylcholine (DSPC), dioleylphosphatidylcholine (DOPC) and cholesterol (CHOL) were purchased from Sigma (St. Louis, MO, USA). Distearoylphosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG2000) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Angiotensin-(1-7) was obtained from Bachem (Los Gatos, CA, USA). All other reagents were of reagent grade and used without further purification.

2.2. Animals

Wistar rats (male weighing 250 ± 10 g), bred at the animal facility of the Biological Sciences Institute (CEBIO, UFMG, Belo Horizonte, MG, Brazil), were used throughout the experiments.

2.3. Preparation and characterization of liposomes

Ang-(1-7) was encapsulated in five different preparations of liposomes (Table 1), using as a first step, the dehydration–rehydration method [6]. Liposomes were either kept uncalibrated or were extruded repeatedly through polycarbonate membrane of 200 nm pore size. The liposomes were then separated from non-encapsulated peptide by dialysis against PBS (0.15 M NaCl, 0.01 M phosphate, pH 7.2) and finally sterilized by filtration through 0.22 μm membranes. Calibrated and uncalibrated empty liposomes were obtained using

Table 1
Characteristics of liposome preparations containing encapsulated Ang-(1-7)

Liposome preparation	Lipid composition (molar ratio)	Peptide to lipid ratio (w/w)
(1) Pegylated, rigid	DSPC:CHOL:DSPE-PEG (5:4:0.3)	0.004
(2) Pegylated, rigid, conc.	DSPC:CHOL:DSPE-PEG (5:4:0.3)	0.009
(3) Pegylated, fluid	DOPC:CHOL:DSPE-PEG (5:4:0.3)	0.004
(4) Conventional, rigid	DSPC:CHOL (5:4)	0.004
(5) Pegylated, rigid, uncal.	DSPC:CHOL:DSPE-PEG (5:4:0.3)	0.013

the same procedure, but omitting the peptide. The amount of encapsulated peptide was determined by exploiting its intrinsic fluorescence. The mean hydrodynamic diameter of the vesicles, as determined by photon correlation spectroscopy, was found to be equal to 200 ± 15 nm for calibrated liposomes.

2.4. Microinjection of liposome preparations and measurements of MAP and HR

Ang-(1-7)-containing liposomes (50 ng of peptide/200 nl for Preparations 1, 3, 4, 5 and 100 ng/200 nl for Preparation 2) or empty liposomes (control group) were unilaterally microinjected into the RVLM of Wistar rats (3–10 animals per group), under tribromoethanol anesthesia (250 mg/kg i.p.), with an injection needle (30 G) that was slowly inserted in the brain tissue through the dorsal surface, using the following extereotaxis coordinates: 1.8 mm anterior, 1.8 mm lateral to the obex and just above the pia-mater. Mean arterial pressure (MAP) and heart rate (HR) were registered by telemetry during 10 s every 10 min, for 4 days before and for up to 12 days after microinjection of the liposome preparations in the undisturbed freely moving animals, as previously described [5].

2.5. Treatment of data and statistical analysis

From telemetry data, daytime and nighttime mean MAP and HR were determined on each day for each animal. The changes in these parameters were then determined by calculating the difference between the value of the parameter on each day and the mean of the parameter over the 3 days preceding microinjection (control period). The values for cardiovascular parameters after microinjection were compared to those before microinjection (control period), using the One-way ANOVA for repeated measures (with Dunnett post test). The changes in cardiovascular parameters were compared between the different experimental groups, using the Two-way ANOVA followed by Bonferroni post test.

3. Results

Ang-(1-7) was encapsulated in different liposomal preparations (Table 1), in order to evaluate the influence of membrane fluidity, membrane surface, liposome size and amount of encapsulated peptide on the cardiovascular effects of encapsulated Ang-(1-7) at the RVLM. Encapsulation of Ang-(1-7) in liposomes was achieved with an efficiency of 15% for preparations calibrated through 200 nm polycarbonate membranes (Preparations 1, 2, 3, 4) and 45% for uncalibrated preparation.

These liposomal preparations were microinjected unilaterally into the RVLM of Wistar rats, and MAP and HR were registered by telemetry in the undisturbed freely moving animals.

Table 2 summarizes the cardiovascular effects elicited by the different liposome preparations, showing the baseline values for MAP and HR during the control period and indicating the number of days during which these cardiovascular parameters were significantly altered when compared to the baseline values.

Microinjection of Ang-(1-7) encapsulated in pegylated, rigid and calibrated liposomes (Preparation 1) produced a significant increase of MAP for 7 days, specifically on the daytime period. Bradycardia was also observed during nighttime ($P < 0.05$ on days 1, 2, 3). Strikingly, none of these effects was observed after microinjection of empty liposomes (Preparation 6) at the same lipid dose. As shown in Table 2, empty liposomes produced daytime tachycardia on day 1, which could be attributed to mechanical stimulation. Fig. 1 displays the changes in MAP and HR elicited by Preparation 1 and the comparison of these effects with those produced by empty liposomes. Significant differences were observed for daytime MAP on days 1 to 7 and for nighttime HR on day 2.

When pegylated, rigid and calibrated liposomes were given at the same lipid dose, but with a two-fold higher peptide dose (Preparation 2), pressor effects of short duration were produced on both daytime and nighttime periods ($P < 0.05$ only on day 2) (Table 2). In this case, no significant alteration of HR was detected. As illustrated in Fig. 2, the changes in MAP promoted by this preparation were significantly different from those elicited by empty liposomes. However, these alterations did not differ significantly from those produced by Preparation 1.

Microinjection of Ang-(1-7) encapsulated in pegylated, fluid and calibrated liposomes (Preparation 3) produced an early pressor effect ($P < 0.05$ on day 1)

Table 2
Chronic cardiovascular actions elicited by Ang-(1-7) at the RVLM from different liposome preparations

Liposome preparation	MAP change		HR change	
	Daytime	Nighttime	Daytime	Nighttime
(1) Pegylated, rigid	↑ ↑ ↑ ↑ ↑ ↑ ↑	ns	ns	↓↓↓
	(101 ± 11)	(109 ± 12)	(301 ± 12)	(357 ± 19)
(2) Pegylated, rigid, conc.	↑	↑	ns	ns
	(96 ± 10)	(99 ± 9)	(325 ± 18)	(372 ± 37)
(3) Pegylated, fluid	↑	↑	ns	↓↓↓↓
	(89 ± 12)	(94 ± 13)	(332 ± 17)	(397 ± 5)
(4) Conventional, rigid	ns	ns	↑	ns
	(94 ± 9)	(100 ± 13)	(306 ± 10)	(349 ± 13)
(5) Pegylated, rigid, uncal.	ns	↑	↑ ↑	ns
	(88 ± 4)	(94 ± 5)	(319 ± 6)	(364 ± 8)
(6) Empty, cal.	ns	ns	↑	ns
	(92 ± 8)	(96 ± 9)	(317 ± 24)	(362 ± 26)
(7) Empty, uncal.	ns	ns	↑ ↑	ns
	(88 ± 13)	(93 ± 13)	(330 ± 15)	(378 ± 20)

The number of sets represents the number of days during which a significant difference of the parameter was observed, when compared to its value just before microinjection (control period). ↑ indicates a significant increase and ↓ indicates a significant decrease of the parameter. "ns" means no significant difference ($P>0.05$, one-way ANOVA for repeated measures). Values between parentheses represent the average values of MAP (in mm Hg) or HR (in bpm) during the control period ± standard deviations.

and a late bradycardia on the nighttime period ($P<0.05$ on days 3, 4, 6, 7) (Table 2). As illustrated in Fig. 3, the changes in MAP and HR promoted by this preparation were significantly higher than those produced by empty liposomes. Moreover, the changes in MAP were significantly lower than those produced by Preparation 1 ($P<0.05$ on day 1, nighttime; $P<0.05$ on day 2, daytime).

It is noteworthy that pegylation of liposomes was found to be required for producing significant alter-

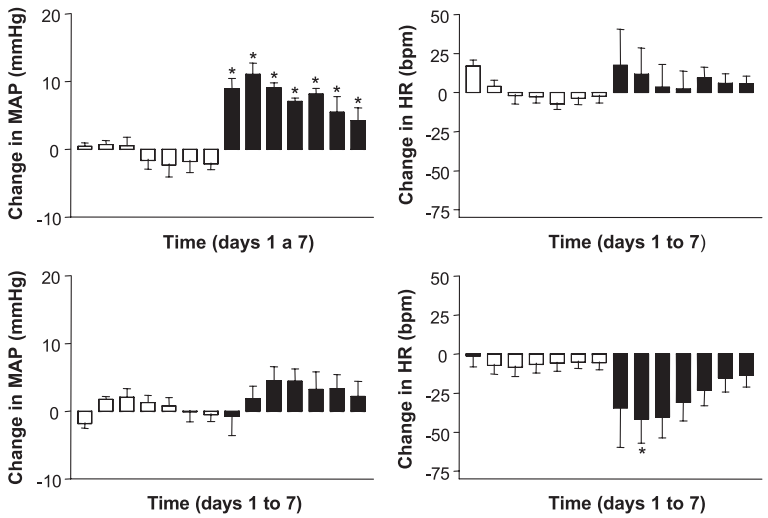

Fig. 1. Daytime (upper) and nighttime (lower) changes in MAP and HR induced by microinjection of pegylated, rigid and calibrated liposomes, containing (filled bars) or not (empty bars) 50 ng of Ang-(1-7), into the RVLM of Wistar rats. Data represent the means of observed changes ± S.E. *$P<0.05$ for comparisons between the changes induced by peptide-containing liposomes ($n=5$) and those induced by empty liposomes ($n=10$) (Two-way ANOVA with Bonferroni post test).

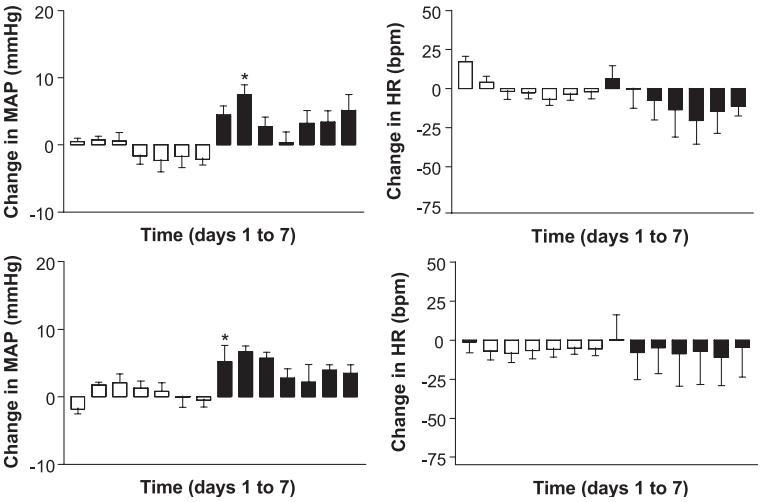

Fig. 2. Daytime (upper) and nighttime (lower) changes in MAP and HR induced by microinjection of pegylated, rigid, concentrated and calibrated liposomes, containing (filled bars) or not (empty bars) 100 ng of Ang-(1-7), into the RVLM of Wistar rats. Data represent the means of observed changes ± S.E. *$P < 0.05$ for comparisons between the changes induced by peptide-containing liposomes ($n = 3$) and those induced by empty liposomes ($n = 10$) (Two-way ANOVA with Bonferroni post test).

ation of cardiovascular parameters, as non-pegylated, rigid and calibrated liposomes (Preparation 4) showed neither pressor effect nor bradycardia (Table 2). The changes in daytime MAP elicited by Preparation 1 were found to be significantly higher than those produced by Preparation 4 ($P < 0.05$ on days 1, 2, 3). Moreover, the changes in nighttime HR elicited by Preparation 3 were significantly higher than those produced by Preparation 4 ($P < 0.05$ on days 3, 4, 6, 7).

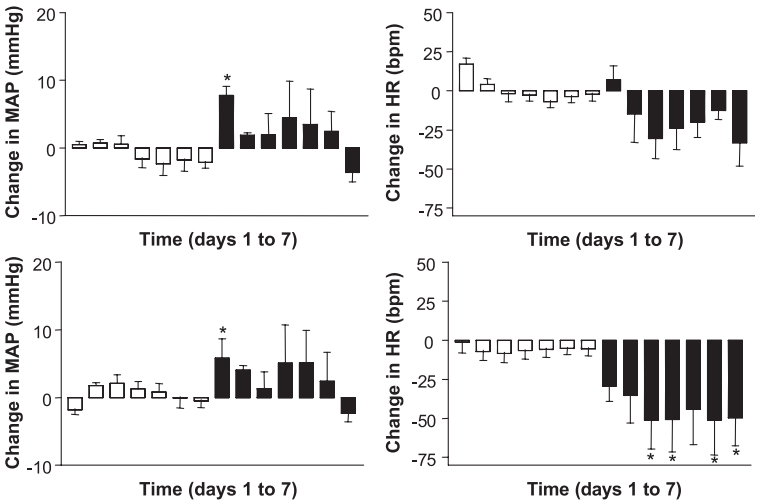

Fig. 3. Daytime (upper) and nighttime (lower) changes in MAP and HR induced by microinjection of pegylated, fluid and calibrated liposomes, containing (filled bars) or not (empty bars) 50 ng of Ang-(1-7), into the RVLM of Wistar rats. Data represent the means of observed changes ± S.E. *$P < 0.05$ for comparisons between the changes induced by peptide-containing liposomes ($n = 3$) and those induced by empty liposomes ($n = 10$) (Two-way ANOVA with Bonferroni post test).

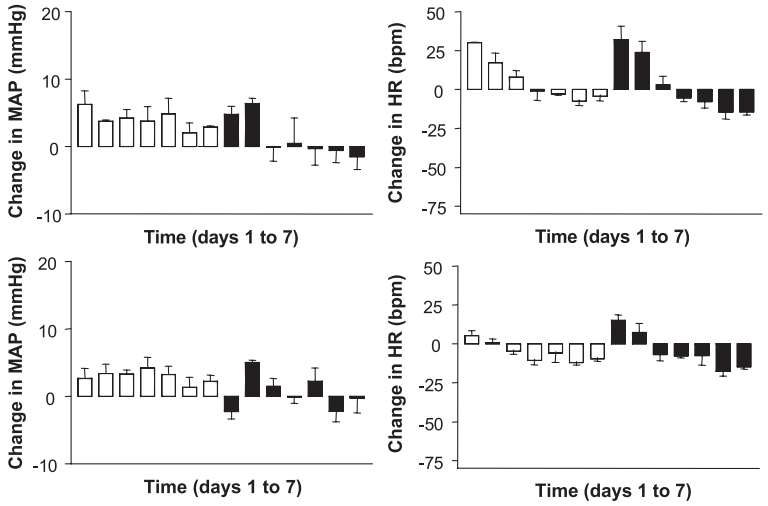

Fig. 4. Daytime (upper) and nighttime (lower) changes in MAP and HR induced by microinjection of pegylated, rigid and uncalibrated liposomes, containing (filled bars) or not (empty bars) 50 ng of Ang-(1-7), into the RVLM of Wistar rats. Data represent the means of observed changes ± S.E. $P > 0.05$ for comparisons between the changes induced by peptide-containing liposomes ($n = 3$) and those induced by empty liposomes ($n = 3$) (Two-way ANOVA with Bonferroni post test).

When pegylated and rigid liposomes were administered in the uncalibrated form (Preparation 5, liposomes with large and heterogeneous size distribution), a significant pressor effect was detected specifically on nighttime ($P < 0.05$ on day 2) (Table 2). However, no significant difference was observed between the change produced by this preparation and that produced by uncalibrated empty liposomes (Fig. 4). The changes in daytime MAP elicited by Preparation 1 were found to be significantly higher than those produced by Preparation 5 ($P < 0.05$ on day 3).

4. Discussion

It has been reported previously that microinjection of Ang-(1-7)-containing liposomes into the RVLM of Wistar rats elicited a prolonged pressor effect for several days, in contrast to the microinjection of empty liposomes or of the free peptide [5]. This long-lasting action was achieved using pegylated, rigid and calibrated (200 nm) liposomes. Moreover, microinjection of the same liposomes labeled with the lipophilic fluorescent dye, diI, showed that vesicles remained concentrated at the RVLM even after 7 days [5].

In the present work, the potential of this new method was further investigated, by evaluating the influence of liposome characteristics on the cardiovascular actions of encapsulated peptide. It is expected that manipulation of vesicle size, membrane surface, membrane fluidity and amount of encapsulated peptide would result in the modulation of the flux of peptide infusion. Ultimately, physiologically relevant information on the relationship between the flux of peptide release and the cardiovascular responses may be obtained.

Pressor effects and bradycardia were the main actions elicited by the liposome preparations at the RVLM. However, the period, duration and intensity of these effects were found to depend markedly on liposome characteristics. The most intense and prolonged effects were produced by pegylated, rigid and calibrated liposomes (Preparation 1). Less pronounced effects were observed when liposomes were uncalibrated (Preparation 5), contained a higher amount of peptide (Preparation 2) or were presented with a fluid membrane (Preparation 3).

The dependence of cardiovascular responses upon liposome characteristics, as evidenced in this study, may be explained by the different release profiles of the liposome preparations. Since the spontaneous release of Ang-(1-7) was found to be insignificant on a week period, when these liposome preparations were resuspended in isotonic saline (data not shown), cell-

mediated release is expected to be the predominant mechanism of peptide release in vivo. The presence of active endocytic cells at the microinjection site, most probably microglial cells [7], is also supported by the observation that pegylation, that essentially slows down the uptake of liposomes by cells [8], was required for producing long-lasting responses. Assuming that fluid liposomes are more susceptible to phospholipase degradation than rigid ones [9], that large (uncalibrated) liposomes are captured more avidly by endocytic cells than smaller ones [10] and that liposomes containing higher amount of peptide exhibit a higher flux of peptide release, one expects indeed that pegylated, rigid and calibrated liposomes would behave as the slowest release system.

From the physio-pharmacological point of view, this study confirms, in chronic conditions, the pressor effect of Ang-(1-7) at the RVLM and unmasks a new action of the peptide on HR. Moreover, it suggests that MAP changes are influenced by HR changes, since bradycardia was usually accompanied by a loss of pressor effect (observed in the case of Preparations 1 and 3) and the pressor effect was usually observed in the absence of bradycardia (in the case of Preparation 2). This data also suggests that cardiovascular responses to Ang-(1-7) strongly depend on the local peptide concentration, the intensity of the pressor effect and the bradycardia being reduced at high concentrations. Such effect may be related to the presence of Ang-(1-7) receptors in both excitatory (glutamatergic-) and inhibitory neurons at the RVLM, as recently described for Ang II [11].

5. Conclusion

This study shows that the cardiovascular responses to the microinjection of Ang-(1-7)-containing liposomes into the RVLM can be modulated by manipulating liposome characteristics, the most intense and prolonged effects being produced by pegylated, rigid and calibrated liposomes. Further insights into the neuromodulator actions of Ang-(1-7) at the RVLM and its role in the central control of blood pressure were obtained. It is expected that this new method will encounter numerous applications in the study of the chronic actions of short-lived bioactive peptides in specific sites of the brain.

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