In vivo antihypertensive mechanism of lactoferrin-derived peptides: reversion of angiotensin I- and angiotensin II-induced hypertension in Wistar rats

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Abbreviations: ACE, angiotensin-I-converting enzyme; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; ECE, endothelin-converting enzyme; LF, lactoferrin; RAS, renin angiotensin system; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat

Abstract

Novel peptides with antihypertensive effects in SHR rats have previously been identified in lactoferrin (LF) hydrolysates. To investigate their *in vivo* antihypertensive mechanism, we have assessed the blood pressure lowering effects of two of these LF-derived peptides (RPYL and DPYKLRP) in Wistar rats subjected to either angiotensin I- or angiotensin II-induced hypertension. Blood pressure was measured by the tail-cuff method, hypertension was induced by subcutaneous infusion of angiotensins, and then captopril, valsartan or LF-derived peptides orally administered. Angiotensin I- and angiotensin II-induced hypertension were reversed by captopril and valsartan, respectively. RPYL and DPYKLRP reversed angiotensin I-induced hypertension, while DPYKLRP but not RPYL produced a modest reversion of angiotensin II-elicited hypertension. Neither RPYL nor DPYKLRP modified normotension. Thus, *in vivo* ACE inhibition is involved in the antihypertensive effects of LF-derived peptides like RPYL and DPYKLRP, while inhibition of AT_1 receptor-mediated vasoconstriction plays a less relevant role.

Keywords:

Antihypertensive peptides

Lactoferrin-derived peptides

Angiotensin-induced hypertension

Wistar rat

In vivo ACE inhibition

Renin angiotensin system

1. Introduction

Hypertension is an important modifiable risk factor for cardiovascular disease, which its management includes not only pharmacological treatment but also lifestyle changes like physical activity and dietary habits (Ruilope, 2011). The increasing perception about the relationship between food and health is fostering the development of functional foods providing health benefits beyond nutrition (Roberfroid, 2002). Some dietary proteins contain embedded peptides that once released behave as bioactive peptides with different health-promoting properties including blood pressure lowering effects (Hartmann & Meisel, 2007).

The renin angiotensin system (RAS), a key player in blood pressure and fluid balance regulation, is one of the main targets for the treatment of hypertension. Its inhibition at three possible levels, angiotensin-converting enzyme (ACE), upstream renin activity, or downstream angiotensin receptors, is the pharmacological basis for commonly used antihypertensive drugs (Fragasso et al., 2012). ACE inhibition is also the most aimed target for antihypertensive food-derived peptides developed as an alternative to drugs (Hong et al., 2008). Although different animal and plant proteins have been used, milk is the main source of antihypertensive ACE-inhibitory peptides reported to date (Hernández-Ledesma, Contreras, & Recio, 2011; Korhonen, 2009).

Despite numerous efforts, the *in vivo* mechanism underlying vasoactive and blood pressure lowering effects of antihypertensive food-derived peptides has not yet been fully established, which may hamper their use as bioactive ingredients in functional foods. A recent Scientific Opinion of the European Food Safety Authority on the substantiation of health claims related to isoleucine-proline-proline (IPP) and valine-proline-proline (VPP) and maintenance of normal blood pressure, stated that there was no convincing evidence for a mechanism by which these widely studied bioactive peptides could exert the claimed effect (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2012). Beyond *in vivo* ACE inhibition reported for some peptides and hydrolysates (Jäkälä, Hakala , Turpeinen , Korpela, &

Vapaatalo, 2009; Lu et al., 2011; Wang et al., 2012; Yang, Yang, Chen, Tzeng, & Han, 2004) antihypertensive effects could be mediated by their interaction with other RAS steps and related pathways in the vascular system, potentially contributing to blood pressure reduction (Udenigwe & Mohan, 2014).

In previous studies, antihypertensive properties of peptides derived from bovine lactoferrin (LF), a well-characterized protein of milk whey, were shown in spontaneously hypertensive rats (SHR) (Ruiz-Giménez et al., 2010). Focusing on the RAS system, we have reported *in vitro* ACE inhibition by a LF pepsin hydrolysate (named pepsin LFH <3kDa) and its antihypertensive effect in SHR rats after acute oral administration (Ruiz-Giménez et al., 2012). Moreover, chronic oral administration of pepsin LFH <3kDa to SHR rats resulted in reductions of hypertension progression, circulating ACE activity, angiotensin II and aldosterone levels, as well as a compensatory increase of renin activity (Fernández-Musoles, Manzanares, Burguete, Alborch, & Salom, 2013a). Recently, we have reported that *in vitro* inhibition of ACE activity by pepsin LFH <3kDa also occurred in cultured human endothelial cells (García-Tejedor et al., 2015). On the other hand, dairy yeasts (*Debaryomyces hansenii*, *Kluyveromyces lactis* and *K. marxianus*) produced LF-derived antihypertensive hydrolysates. Among them, the hydrolysate produced by a particular strain of *Kluyveromyces marxianus* (named Km2 pLFH) showed the highest *in vitro* ACE inhibition and *in vivo* blood pressure reduction in SHR rats (García-Tejedor, Padilla, Salom, Belloch, & Manzanares, 2013).

Novel bioactive peptides have been identified in LF hydrolysates obtained by enzymatic proteolysis or yeast fermentation, and some of them have been particularly characterized. Among peptides identified in pepsin LFH <3kDa, the tetrapeptide RPYL produced *in vitro* ACE inhibition, *ex vivo* inhibition of ACE-dependent vasoconstriction induced by angiotensin I and *in vivo* reduction of systolic blood pressure in SHR rats (Ruiz-Giménez et al., 2012). Moreover, RPYL also produced *ex vivo* inhibition of angiotensin II-elicited vasoconstriction by blocking angiotensin AT_1 receptors (Fernández-Musoles et al., 2014). On

the other hand, among peptides identified in Km2 pLFH, the heptapeptide DPYKLRP produced *in vitro* ACE inhibition and *in vivo* decrease of systolic blood pressure in SHR rats. Moreover, antihypertensive effects in SHR rats were accompanied by reductions in circulating ACE activity and angiotensin II level (García-Tejedor et al., 2014). Further *in vivo* studies to elucidate the mechanism of action of milk protein-derived antihypertensive peptides are still necessary to develop dairy functional foods. In order to gain insight into the *in vivo* antihypertensive mechanism of the LF-derived peptides RPYL and DPYKLRP, we have assessed their blood pressure lowering effects in Wistar rats subjected to either angiotensin I- or angiotensin II-induced hypertension.

2. Materials and methods

2.1. Materials

Peptides (RPYL and DPYKLRP) were ordered from GenScript Corp. (Piscataway, NJ, USA) wherein they were synthesized by solid phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. Peptide purities of supplied batches were 99.7% for RPYL and 96.9% for DPYKLRP. Angiotensin I, angiotensin II, captopril and valsartan were purchased from Sigma-Aldrich Química (Tres Cantos, Madrid, Spain). ALZET Osmotic Pumps (model 2ML4) were purchased from Charles River Laboratories (Barcelona, Spain). Diazepam and ketamine were purchased from Roche Farma (Madrid, Spain) and Parke-Davis (Alcobendas, Madrid, Spain), respectively.

2.2. Animal welfare

Experimental procedures were conducted in accordance with the Spanish legislation on 'Protection of Animals used for Experimental and other Scientific Purposes' and the study was approved by the 'Ethics Committee for Animal Welfare' of the Hospital La Fe to be carried out in its accredited animal research facility.

Ten male Wistar rats (200-225 g) were supplied by Charles River Laboratories (Barcelona, Spain). Rats were housed in temperature-controlled rooms (23 °C) with 12 h light/dark cycles, and consumed tap water and standard diet *ad libitum*. A two-week period of acclimatization was allowed to recover from the stress associated with transportation (Obernier & Baldwin, 2006). To minimize the impact of light cycle and feeding on circadian rhythms of blood pressure (van den Buuse, 1999), the experiments always started at the same time in the morning (9:00 a.m.) in fasted rats.

2.3. Blood pressure measurement

Indirect measurement of systolic blood pressure (SBP) was carried out in awake restrained rats by the noninvasive tail-cuff method using computer-assisted Non-Invasive Blood Pressure equipment (LE5001 unit with LE5160R cuff and transducer, Panlab Harvard Apparatus, Cornellá, Barcelona, Spain). This method has been validated with direct intra-arterial measurements (Ibrahim, Berk, & Hughes, 2006). Before the measurements, rats were kept at 37 °C during 15 min to make the pulsations of the tail artery detectable. Each value of SBP was obtained by averaging at least three consecutive and successful measurements without disturbance of the signal. Changes in SBP were calculated as the absolute difference (in mm Hg) with respect to the basal values of measurements obtained just before starting the treatments.

2.4. Hypertension induction

Rats were anaesthetized by intraperitoneal injection of 5 mg/kg diazepam and 100 mg/kg ketamine. An ALZET Osmotic Pump (model 2ML4) was surgically implanted subcutaneously on the back, between and slightly posterior to the scapulae. The osmotic pump was filled with either angiotensin I (11.1 mg/2 mL 0.1 M acetic acid) or angiotensin II (11.1 mg/2 mL distilled water), and delivered continuously for 4 weeks at a rate of 2.5 µL/hr, that is around 1 mg angiotensin/kg/day. The SBP was measured before angiotensin infusion

(zero time) and twice a week during 24 days of infusion. Physiological saline was infused as negative control.

2.5. Assay of lactoferrin-derived peptides

Peptides (RPYL or DPYKLRP, 10 mg/kg) were orally administered by gastric intubation in 650 µL of physiological saline. The SBP was measured before peptide intake (zero time) and 1.5, 3, and 24 h after intake. In assays on angiotensin I-induced hypertension captopril (10 mg/kg) served as positive control, whereas in assays on angiotensin II-induced hypertension valsartan (10 mg/kg) was the positive control. The vehicles for peptides and captopril (physiological saline) and for valsartan (dimethyl sulfoxide, DMSO) were assayed as negative controls.

2.6. Data analysis and statistics

Values are expressed as the mean ± SEM. Unpaired Student's t test was used to assess differences between two groups. Analysis of variance (ANOVA) followed by Student-Newman-Keuls test or Dunnett's test was used for multiple comparisons among more than two groups. P values <0.05 were considered significant.

3. Results

3.1. *Angiotensin I and angiotensin II induced blood pressure increases*

Average basal SBP in Wistar rats was 119 ± 2 mm Hg (n = 10). Continuous subcutaneous infusion of angiotensin I (1 mg/kg/day) induced increase in SBP (Fig. 1), which became significantly higher than SBP in saline-infused control rats from day 3. Steady-state SBP levels were attained after 17 days (151 ± 7 mm Hg, n = 4 in angiotensin I *vs* 123 ± 1 mm Hg, n = 3 in saline control at this time point). Infusion of angiotensin II (1 mg/kg/day) induced increase in SBP (Fig. 1), which became significantly higher than SBP in both saline control- and angiotensin I-infused rats from day 3. Steady-state SBP levels were also attained after 17 days (189 ± 1 mm Hg, n = 3 in angiotensin II *vs* 123 ± 1 mm Hg, n = 3 in saline control at this time

point). SBP remained at hypertensive levels in both angiotensin I- and angiotensin II-infused rats until the end of infusion, at day 28, and returned to normotensive values upon osmotic pump withdrawal (126 ± 1 mm Hg, n = 4; and 123 ± 1 mmHg, n = 3, respectively).

3.2. Lactoferrin-derived peptides reversed angiotensin I-induced hypertension

In angiotensin I-induced hypertensive rats (155 ± 2 mm Hg, n = 26), oral boluses (10 mg/kg) of RPYL or to a higher extent DPYKLRP induced transient decreases in SBP. Values of SBP were maximally reduced at 1.5 h after peptide intake, remained significantly reduced at 3 h, and returned to basal values at 24 h (Fig. 2). For comparison, oral captopril (10 mg/kg) elicited decrease in SBP which was maximal at 1.5 h after intake and remained significant even at 24 h (Fig. 2). Decreases in SBP induced by the two peptides and captopril at 1.5 h were significantly different among them: RPYL < DPYKLRP < captopril ($P<0.05$, Student-Newman-Keuls test).

3.3. Heptapeptide DPYKLRP but not tetrapeptide RPYL reversed angiotensin II-induced hypertension

With regard to angiotensin II-induced hypertensive rats (183 ± 2 mm Hg, n = 34), oral boluses (10 mg/kg) of DPYKLRP but not RPYL induced transient decrease in SBP. Values of SBP were significantly reduced only at 1.5 h after DPYKLRP intake, and returned to basal values at 24 h (Fig. 3). For comparison, oral valsartan (10 mg/kg) elicited decrease in SBP (when compared to DMSO control) which was maximal at 1.5 after intake and remained significant even at 24 h (Fig. 3). In contrast, captopril (10 mg/kg) did not significantly modify SBP level in angiotensin II-induced hypertensive rats (Fig. 3).

3.4. Lactoferrin-derived peptides did not modify normotensive blood pressure levels

Neither lactoferrin-derived peptides (RPYL and DPYKLRP) nor drugs (captopril and valsartan), orally administered at the same dose used in hypertensive rats (10 mg/kg), produced significant changes on normortensive SBP levels in Wistar rats (Table 1).

4. Discussion

We have found that both the tetrapeptide RPYL and to a higher extent the heptapeptide DPYKLRP reverse angiotensin I-induced hypertension when orally administered to Wistar rats. Moreover, DPYKLRP also produces a modest reversion of angiotensin II-elicited hypertension. Of note, neither RPYL nor DPYKLRP modified arterial blood pressure in normotensive rats. We and others have extensively used the SHR rat as hypertension model to assess the antihypertensive effects of food protein-derived bioactive peptides (Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012). However, this approach does not allow knowing the *in vivo* antihypertensive mechanism, unless for example blood components of the RAS are determined (Fernández-Musoles et al., 2013a; Lu et al., 2011). Alternatively, angiotensin I- or angiotensin II-induced hypertension rat models have been used to gain insight into the *in vivo* antihypertensive mechanism of diverse non-drug natural products (Liu et al., 2003; Prasad, 2013; Waghulde, Mohan, Kasture, & Balaraman, 2010). In the present study, we have combined both angiotensin I- and angiotensin II-induced hypertension in order to discriminate between effects on ACE activity and effects on downstream activation of angiotensin AT_1 receptors of LF-derived peptides.

Antihypertensive effects in SHR rats have previously been shown for both RPYL (Ruiz-Giménez et al., 2012) and DPYKLRP (García-Tejedor et al., 2014). In the present study, both RPYL and DPYKLRP reversed angiotensin I-induced hypertension in Wistar rats. Like in SHR rats, the magnitude and duration of the antihypertensive effect was higher for DPYKLRP than for RPYL on angiotensin I-induced hypertension. We used the ACE inhibitor drug captopril as a positive control, which showed antihypertensive effects. It has been previously reported that captopril, at the same dose used in our study, produces almost complete inhibition of plasma ACE activity and attenuation of pressor responses to angiotensin I (Levens, Peach, Vaughan, Weed, & Carey, 1981). Therefore, angiotensin I-induced hypertension is a suitable model to assess *in vivo* ACE inhibition, and then our results support ACE inhibition as antihypertensive

mechanism for the LF-derived peptides RPYL and DPYKLRP. Few studies have previously assessed *in vivo* ACE inhibitor effect of milk-derived peptides by means of angiotensin I administration. Milks fermented using two strains of *Lactobacillus helveticus* produced inhibition of angiotensin I-elicited acute pressor responses in anaesthetized Sprague Dawley rats (Fuglsang, Rattray, Nilsson, & Nyborg, 2003b). In contrast, milk-derived short peptides produced no effect or very moderate inhibition of these angiotensin I-elicited pressor responses (Fuglsang, Nilsson, & Nyborg, 2003a) On the other hand, a peptide concentrate obtained from hydrolysis of bovine whey brought about by *Cynara cardunculus* cardosins (PepC) and an α-lactalbumin-derived peptide identified in PepC (KGYGGVSLPEW) produced inhibition of angiotensin I-elicited acute pressor responses in anaesthetized SHR rats (Tavares, Sevilla, Montero, Carrón, & Malcata, 2012). In contrast to these previous studies in anesthetized rats, our *in vivo* assays were carried out in awake, shortly restrained rats, with a steady-state level of angiotensin I-induced hypertension, which resembles established hypertension in SHR rats.

We have previously shown that antihypertensive LF-derived peptides induce some changes in blood RAS components of SHR rats. On one hand, the pepsin LFH <3kDa hydrolysate in which RPYL was identified induced reductions of circulating ACE activity, angiotensin II and aldosterone levels, as well as a compensatory increase of renin activity (Fernández-Musoles et al., 2013a). On the other hand, DPYKLRP also induced reductions of circulating ACE activity and angiotensin II level (García-Tejedor et al., 2014). Thus, reversions of angiotensin I-induced hypertension observed in the present study are in line with reported changes in blood RAS components, and all together consistently support ACE inhibition as *in vivo* antihypertensive mechanism for LF-derived peptides like RPYL and DPYKLRP.

As for alternative antihypertensive mechanisms beyond ACE inhibition, our prior *ex vivo* study carried out in isolated arteries showed inhibitory effects of pepsin LFH <3kDa hydrolysate and several LF-derived peptides including RPYL on angiotensin II-induced

vasoconstriction because of an angiotensin AT_1 receptor blocking effect (Fernández-Musoles et al., 2014). This prompted us to assess in the present study the *in vivo* effects of the LF-derived peptides RPYL and DPYKLRP on activation of angiotensin AT_1 receptors. Our results showed that DPYKLRP produced a modest reversion of angiotensin II-elicited hypertension. We used the angiotensin AT_1 receptor antagonist drug valsartan as positive control, which showed strong antihypertensive effects, as previously reported (Kobayashi, Imanishi, & Akasaka, 2006). In contrast, the ACE inhibitor drug captopril did not modify angiotensin II-elicited hypertension, as expected (Textor, Brunner, & Gavras, 1981). Therefore, angiotensin II-induced hypertension is a proper model to assess *in vivo* inhibition of angiotensin AT_1 receptors, and then our results suggest a less relevant role for inhibitory effect on vasoactive responses mediated by angiotensin AT_1 receptors as antihypertensive mechanism for the assayed LF-derived peptides. The fact that *ex vivo* angiotensin AT_1 receptor blocking effect of RPYL was not confirmed in the present *in vivo* study points to oral peptide bioavailability and raises the question about the final active form of food derived antihypertensive peptides (Hernández-Ledesma et al., 2011). As far as we know, no study has previously assessed *in vivo* effects of milk-derived peptides on vasoactive responses mediated by angiotensin AT_1 receptors by means of angiotensin II administration.

Finally, beyond their effects on different steps of the RAS system, we have reported that some LF-derived peptides different to those studied here may act as dual vasopeptidase inhibitors since, in addition to ACE, they can also produce *in vitro* inhibition of endothelin-converting enzyme (ECE) activity and *ex vivo* inhibition of ECE-dependent vasoconstriction (Fernández-Musoles et al., 2010, 2013b). Since ECE is a key enzyme of the endothelin system, which is also involved in vascular tone and blood pressure regulation, *in vivo* participation of this inhibitory mechanism in the antihypertensive effects of LF-derived peptides like RPYL and DPYKLRP deserves further research.

5. Conclusions

Reversion of angiotensin I-induced hypertension by RPYL and DPYKLRP point to *in vivo* ACE inhibition as a mechanism involved at least in part in the antihypertensive effects of these LF-derived peptides. On the other hand, slight reversion of angiotensin II-induced hypertension by DPYKLRP, and no effect at all of RPYL, suggests a less relevant role for an inhibitory effect on vasoactive responses mediated by angiotensin AT_1 receptors as antihypertensive mechanism for these bioactive peptides. Finally, it should be also noted that the effect of RPYL and DPYKLRP were specific to the angiotensin-induced hypertensive states, because these peptides did not modify the arterial blood pressure of normotensive rats. Individual peptides could be applied as nutraceuticals in health-promoting functional foods for the treatment of hypertension.

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Fig. 1 - Time course of systolic blood pressure (SBP) during subcutaneuous continuous infusion (1 mg/kg/day) of angiotensin I and angiotensin II to Wistar rats by means of an osmotic pump. Physiological saline was infused as negative control. SBP is expressed in mm Hg and values are mean ± SEM from 3-4 determinations. *P<0.05 versus saline control, **P<0.01 versus saline control, and ##P<0.01 versus angiotensin I (one-way ANOVA followed by Student-Newman-Keuls tests).

Fig. 2 - Time course of systolic blood pressure (SBP) after oral boluses (10 mg/kg) of lactoferrin-derived peptides RPYL and DPYKLRP administered to angiotensin I-induced hypertensive Wistar rats. Captopril was administered as positive control, whereas the vehicle for peptides and captopril (physiological saline) served as negative control. SBP change (ΔSBP) is expressed in absolute values (mm Hg) and data are mean ± SEM from 4-8 determinations. *P<0.05 versus saline control, **P<0.01 versus saline control (one-way ANOVA followed by Dunnett's tests).

Fig. 3 - Time course of systolic blood pressure (SBP) after oral boluses (10 mg/kg) of lactoferrin-derived peptides RPYL and DPYKLRP administered to angiotensin II-induced hypertensive Wistar rats. Valsartan was administered as positive control, whereas captopril served to check for angiotensin converting enzyme inhibition. The vehicles for peptides and captopril (physiological saline) and for valsartan (dimethyl sulfoxide, DMSO) were administered as negative controls. SBP change (ΔSBP) is expressed in absolute values (mm Hg) and data are mean ± SEM from 4-6 determinations. **P<0.01 versus saline control (one-way ANOVA followed by Dunnett's tests), ##P<0.01 versus DMSO control (unpaired Student's t test).

Table

Table 1. Time course of systolic blood pressure (SBP) in normotensive Wistar rats after oral administration of lactoferrin-derived peptides (RPYL and DPYKLRP), captopril and valsartan.

	Time (h)			
	0	1.5	3	24
Saline[a] **control**	124 ± 1 (6)	125 ± 1 (6)	126 ± 1 (6)	125 ± 2 (6)
Captopril (10 mg/kg)	127 ± 2 (5)	126 ± 2 (5)	125 ± 1 (5)	124 ± 2 (5)
RPYL (10 mg/kg)	126 ± 3 (5)	125 ± 2 (5)	129 ± 2 (5)	127 ± 3 (4)
DPYKLRP (10 mg/kg)	124 ± 2 (6)	125 ± 1 (6)	125 ± 1 (6)	126 ± 1 (6)
DMSO[b] **control**	127 ± 2 (6)	128 ± 2 (5)	127 ± 1 (5)	127 ± 2 (5)
Valsartan (10 mg/kg)	125 ± 3 (3)	127 ± 1 (3)	124 ± 2 (3)	124 ± 1 (3)

SBP is expressed in mm Hg and values are mean ± SEM from (n) determinations.

[a]Vehicle for captopril, RPYL and DPYKLRP.

[b]Dimethyl sulfoxide, vehicle for valsartan.

Figure 1

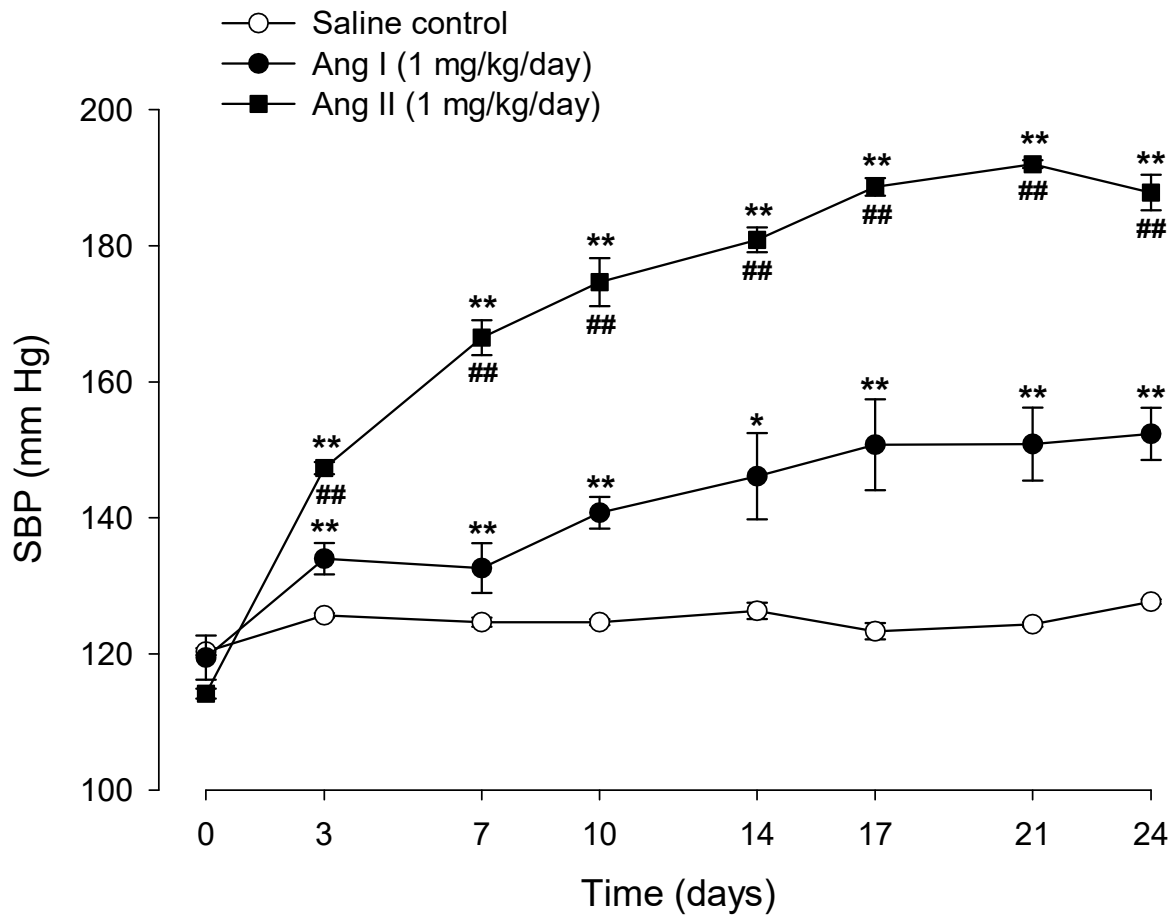

Saline control
Ang I (1 mg/kg/day)
Ang II (1 mg/kg/day)
SBP (mm Hg)
200
180
160
140
120
100
0
3
7
10
14
17
21
24
Time (days)
**
##
*

Figure 2

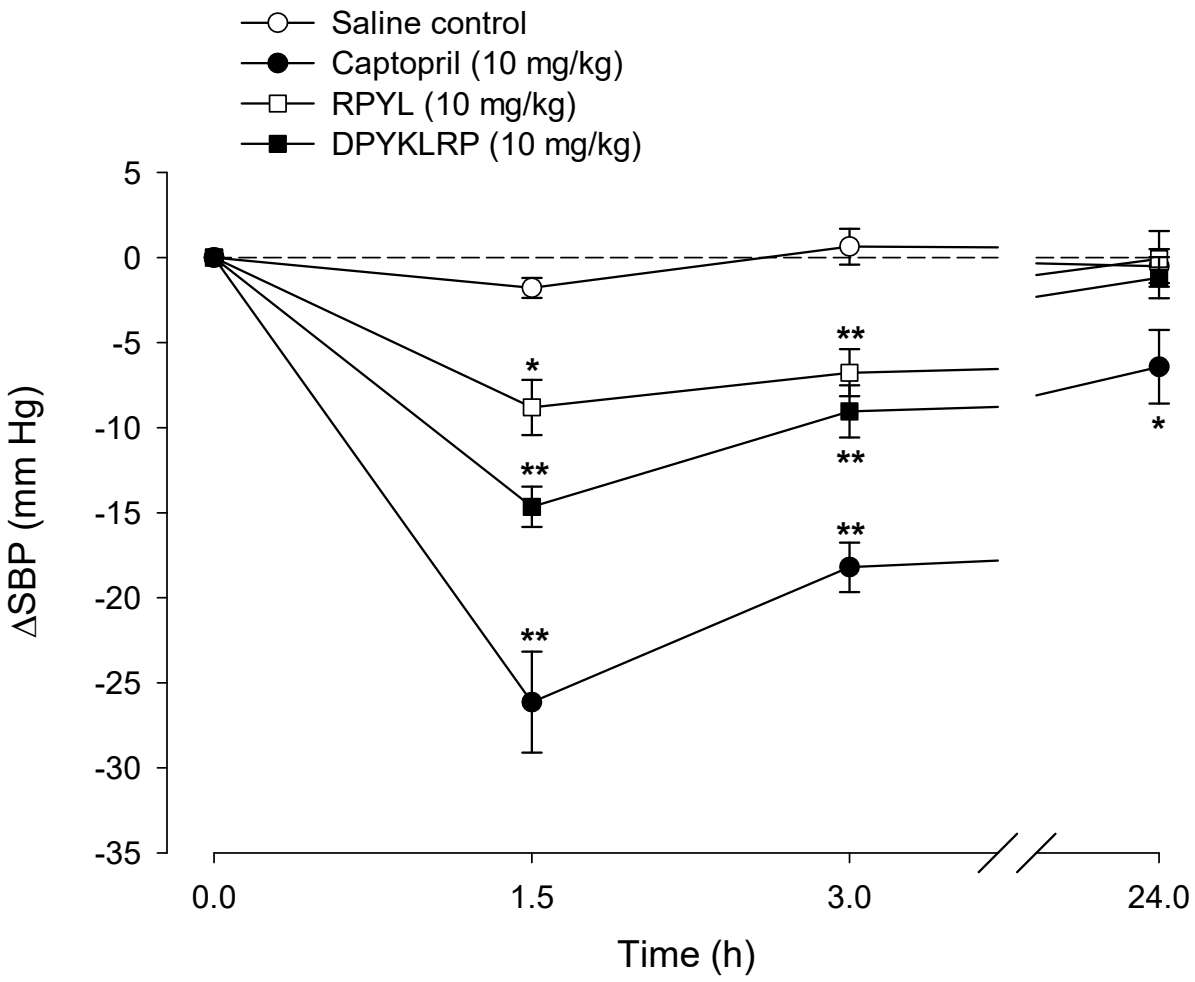
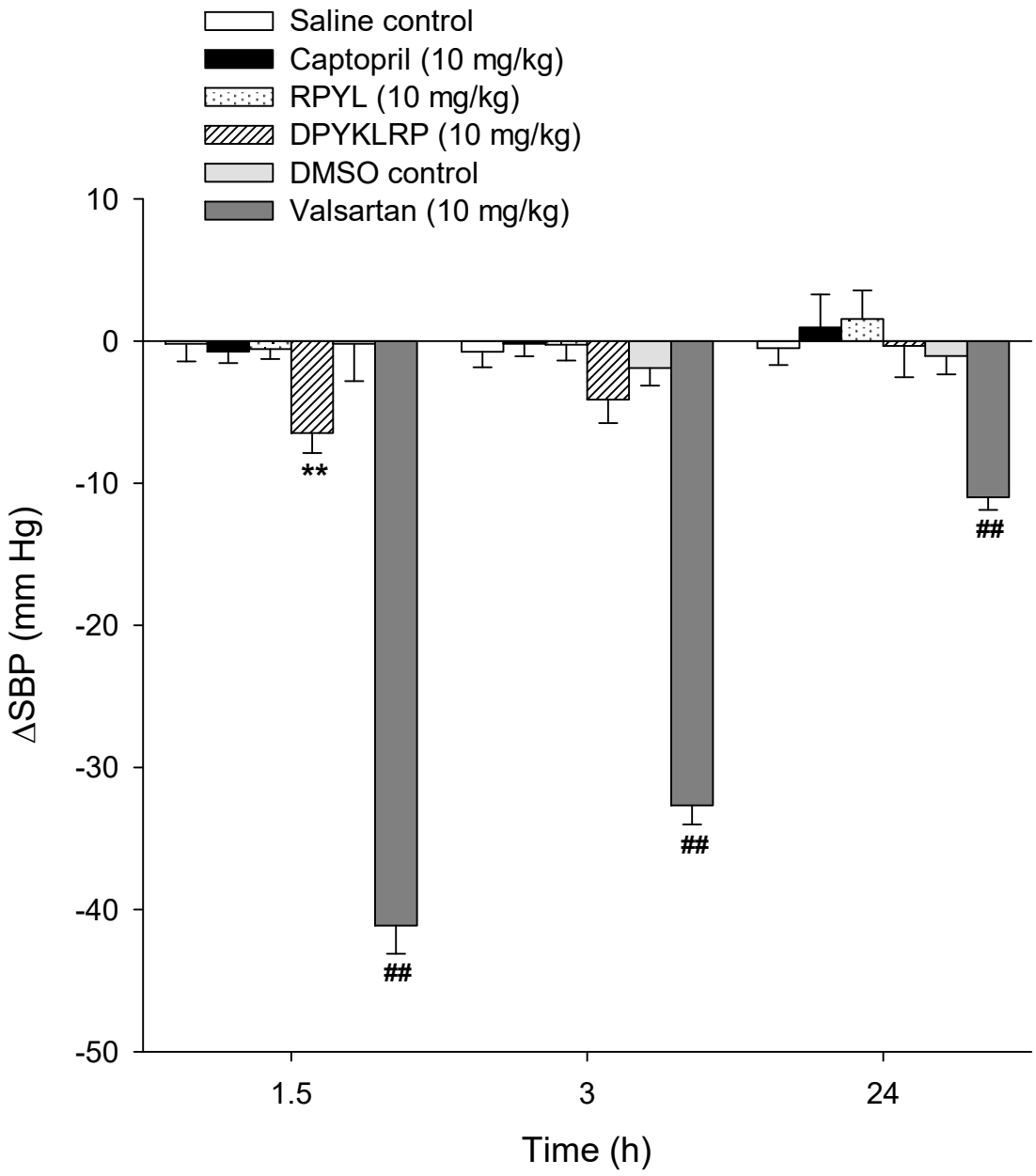

Saline control
Captopril (10 mg/kg)
RPYL (10 mg/kg)
DPYKLRP (10 mg/kg)
5
0
-5
-10
-15
-20
-25
-30
-35
ΔSBP (mm Hg)
0.0
1.5
3.0
24.0
Time (h)
*
**
**
**
**
**
*

Figure 3


Saline control
Captopril (10 mg/kg)
RPYL (10 mg/kg)
DPYKLRP (10 mg/kg)
DMSO control
Valsartan (10 mg/kg)
10
0
-10
-20
-30
-40
-50
ΔSBP (mm Hg)
**
##
##
##
1.5
3
24
Time (h)