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Different blood pressure responses to opioids in three rat hypertension models: role of the baseline status of sympathetic and renin-angiotensin systems

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ABSTRACT

Opioids interact with sympathetic and renin-angiotensin systems in control of arterial pressure (MAP). Our earlier finding that biphalin, a synthetic enkephalin analogue, decreased MAP in anaesthetized spontaneously hypertensive rats (SHR) prompted us to further explore this action, to get new insights into pathogenesis of various forms of hypertension. Biphalin effects were studied in SHR, uninephrectomized rats on high-salt diet (HS/UNX), and rats with angiotensin-induced hypertension (Ang-iH). Beside MAP, renal and iliac blood flows (RBF, IBF) and vascular resistances were measured. In anaesthetized and conscious SHR biphalin, 300 µg h⁻¹kg⁻¹ i.v., decreased MAP by ~10 and ~20 mmHg, respectively (P<0.001). In anaesthetized HS/UNX and normotensive rats MAP increased ~6-7 mmHg (P<0.02); without anaesthesia only transient decreases occurred. MAP never changed in Ang-iH rats. Morphine, 1.5 mg h⁻¹kg⁻¹ i.v., decreased MAP in HS/UNX but only transiently so without anaesthesia, such anaesthesia dependence of response was also seen in normotensive rats. Ang-iH rats never responded to morphine. Hypotensive effect in SHR only depends primarily on the reduction by biphalin of vascular responsiveness to sympathetic stimulation; such increase is well documented for SHR. No MAP response to biphalin or morphine in Ang-iH could depend on angiotensin-induced alterations of the vascular wall morphology and function.

Key-words: arterial blood pressure, biphalin, morphine, peripheral vascular resistance, rat hypertension models, sympathetic nervous system, renin-angiotensin system
INTRODUCTION

In spite of many attempts, no convincing evidence was found to support engagement of the endogenous opioid system in physiological regulation of blood pressure (BP), on the other hand, native and synthetic opioids were reported to exert cardiovascular effects, also on arterial BP, especially when applied directly to the brain (Feuerstein and Siren 1987; Siren and Feuerstein 1992; Sun et al. 1995). In general, in experimental studies brain opioid systems were reported to exert hypertensive or hypotensive influence via stimulation or inhibition of central sympathetic output, the effect depending on the actual brain structures and the opioid receptor species involved (Appel et al. 1986; Siren and Feuerstein 1992). For instance, one early study suggested that enhancement by morphine of Ang II dependent pressor effects was mediated by stimulation of circumventricular organs (probably area postrema) (Shilagyi and Ferrario 1981), and chronic inhibition of opioid receptors with naloxone was reported to suppress the development of hypertension in young SHR (Quock et al. 1984). On the other hand, other workers did not find convincing evidence on the role of brain opioid systems in experimental hypertension (Sun et al. 1996).

Our recent study with SHR demonstrated hypotensive action of intravenous biphalin, a synthetic enkephalin analogue with almost equal affinity for \(\mu\) and \(\delta\) receptors (Misicka et al. 1997), a drug with only minimal physical dependence liability and potentially non-addictive (Yamazaki et al. 2001). While intravenously administered biphalin can cross the blood-brain barrier (BBB) and enter CNS structures engaged in vasomotor control (Abbruscato et al 1997), the drug’s rapid onset of action shown in our preliminary study (Bądzyńska et al. 2016) strongly suggested that stimulation of peripheral opioid receptors was responsible for the pressure decrease. This finding added to the limited evidence indicating that systemic vasodilatation and reduction of elevated blood pressure can be obtained with stimulation of opioid receptors located outside the brain (Cox 1988). Since SHR, a model with pronounced
sympathetic hyperactivity component (Lerman et al. 2005; Pinterova et al. 2011) resembles in many respects essential hypertension in humans, antihypertensive effectiveness of biphalin in this rat strain was of special interest.

One purpose of this study was to examine if biphalin’s hypotensive action could be reproduced in rat hypertension models essentially different from the SHR; we thought that such comparative studies could throw some new light on the pathogenesis of different animal models of hypertension and possibly of different forms of human disease. Second, we examined if the remarkable association of biphalin-induced BP decrease and maintenance or increase of renal perfusion, as observed earlier in SHR (Bądzyńska et al. 2016), could be demonstrated in other models of hypertension. Still another purpose was to find out if depressor effects of biphalin can be reproduced in conscious animals and thus could be regarded directly relevant to animal and human pathophysiology.

Compared here are the responses to intravenous biphalin, an enkephalin analogue which appears to act primarily by stimulation of peripheral opioid receptors, and to morphine which induces both peripheral and central effects, in SHR and two essentially different hypertension models. The first one was developed by exposure of normotensive uninephrectomized rats to high salt intake (HS/UNX), which entails suppression of circulating renin-angiotensin system (RAS). The other model was angiotensin-induced hypertension (Ang-iH) obtained by chronic infusion of subpressor doses of the peptide, a procedure which induces a complex pattern of changes of both RAS and sympathetic nervous system (SNS) (Erdos et al. 2006; Pinterova et al. 2011; Zou et al. 1996). Assuming that the hypotensive response to opioids depends primarily on a decrease in peripheral vascular resistance, we thought it important to examine the involvement of individual vascular beds. Therefore effects of biphalin and morphine on both renal and hind limb vascular resistances were measured in parallel.
MATERIAL AND METHODS

The experimental procedures were approved by the extramural IV Local Ethical Committee for Animal Experimentation, Warsaw. The aim was to compare acute cardiovascular effects of biphalin, a synthetic peripherally acting opioid, and morphine in three models of rat hypertension and in normotensive controls. Two experimental series were made, using male anaesthetized (Series I) and conscious rats (Series II).

Experiments were performed with normotensive Sprague-Dawley (S-D) rats and three models of rat hypertension:

1. Male S-D rats weighing 320-345 g, aged 12 weeks, were subjected to right-side nephrectomy (UNX) and then placed on high-salt (HS) diet (4% Na w/w, Labofeed, Kcynia, Poland) for 14 days. Such procedure (HS/UNX) was previously shown to result in the development of hypertension (Bądzyńska and Sadowski 2012; Fujita et al. 2012).

2. Male S-D rats weighing 280-340 g received continuous infusion of angiotensin II at a dose that was directly subpressor. Alzet osmotic minipumps (model 2002, Durect Co, Cupertino, CA, USA), implanted subcutaneously, were used to deliver Ang II (Sigma-Aldrich, Basel, Switzerland) at 350 µg kg⁻¹ day⁻¹ for 14 days. This dosage induced mild hypertension after about two weeks (angiotensin-induced hypertension, Ang-iH).

3. The third model studied were male spontaneously hypertensive rats (SHR), aged 10-12 weeks, weighing 270-300 g, with established hypertension, derived from the Animal House of Medical University of Warsaw. This group was included here to test reproducibility of earlier results (Bądzyńska et al. 2016) in SHR that were studied at another season of the year (four months difference) and provide data suitable to be directly compared with conscious SHR of the same origin (series II).

Series I: Studies with anaesthetized rats

All the rats were anaesthetized with thiopental (Sandoz GmbH, Kundl, Austria), 100 mg
kg\(^{-1}\) i.p., which provided stable anaesthesia for at least 3.5 h. Mean arterial blood pressure (MAP) and heart rate (HR) were measured via a right femoral artery catheter connected with a Stoelting blood pressure meter (Stoelting, Wood Dale IL, USA). To measure hind limb perfusion (i.e. iliac blood flow, IBF), the area of aortic bifurcation was exposed from a suprapubic incision and a cuff Transonic probe was placed on the iliac artery and connected with a Transonic flowmeter (Type T106, Transonic System Inc. Ithaca, NY, USA). The left kidney was exposed from a subcostal flank incision and immobilized in a plastic holder. Another Transonic cuff probe was placed on the renal artery for measurement of total renal blood flow (RBF). The renal and iliac (hind limb) vascular resistances (RVR, IVR) were calculated as MAP-to-RBF ratio and expressed as mmHg (ml min\(^{-1}\))\(^{-1}\).

**Biphalin studies.** A uniform protocol was used in rats with three models of hypertension described above and in a group of normotensive S-D controls. After saline solvent control, biphalin hydrochloride in saline was infused i.v. at 300 µg h\(^{-1}\)kg\(^{-1}\) BW during 30 min, followed by solvent recovery measurements. We showed that this was the smallest dose that consistently decreased MAP in SHR. The rat groups studied were:

1. HS/UNX rats (\(n = 11\))
2. Ang-iH rats (\(n = 8\))
3. Normotensive S-D rats (\(n = 15\)), a control for groups (1) and (2).

In additional experiments with normal S-D rats (\(n = 6\)), in the 20\(^{th}\) min of biphalin infusion (which was continued throughout the observation period), peripheral opioid receptors were blocked with naloxone methiodide (Sigma, Basel, Switzerland), injected i.v. at a dose of 200 µg kg\(^{-1}\) BW. We found previously that this dose reversed the hypotensive effect of biphalin in SHR but did not affect blood pressure when given alone (Bądzyńska et al. 2016).

4. SHR (\(n = 8\)).

In an additional group of SHR (\(n = 6\)) the naloxone test was performed as described in (3).
Morphine studies. These were conducted with HS/UNX rats \((n = 10)\), Ang-iH rats \((n = 11)\), and in normotensive S-D rats \((n = 12)\) in the way described for biphalin studies. Morphine (Polfa, Warsaw, Poland) in saline was infused i.v. at 1.5 mg h\(^{-1}\) kg\(^{-1}\) BW during 30 min. This was the smallest dose that was previously found to consistently decrease MAP in SHR (Bądzyńska et al. 2016). Since compared to biphalin the effects of morphine were usually delayed but relatively long-lasting, recovery observations were prolonged to at least 30 min after cessation of morphine infusion.

In additional experiments with SHR, naloxone tests for blockade of the early effect of morphine \((n = 11)\) were conducted. At the time when a distinct post-morphine decrease in MAP was attained, usually 10 min after the start of infusion, naloxone methiodide was injected while morphine infusion was continued.

Series II: Studies with conscious rats

Preparatory procedures. For measurement of arterial pressure, and heart rate (HR), in rats of the three experimental hypertension models telemetry probes consisting of a battery-operated radiotransmitter and a catheter (type TA11PA-C40, Data Science International, St. Paul, Minnesota, USA) were implanted 5 days before the start of measurements. Under aseptic conditions and sodium pentobarbital anaesthesia \((50 \text{ mg kg}^{-1} \text{i.p.})\) the abdominal aorta was exposed and the catheter was inserted by direct puncturing of the vessel. The transmitter was placed inside the peritoneal cavity and fixed to the abdominal muscle wall.

In addition, to enable saline solvent and drug infusion, the jugular vein was cannulated with a flexible silicone rubber catheter filled with heparin solution, the catheter was then passed under the skin and the outlet was exposed in the interscapular region, to be later connected with the infusion system. After operation an analgetic drug (Metacam, Leverkusen, Germany; 0.2 mg kg\(^{-1}\) s.c.) and an antibiotic (Baytril 2.5%, Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany; 0.2 mg kg\(^{-1}\) s.c.) were administered. When wound healing was
complete, the rats were habituated to stay in cages that were equipped with an automatic system which enabled programmed i.v. infusion of fluids (Bioanalytical Systems Inc, West Lafayette, Indiana, USA) together with telemetric continuous measurement of MAP and HR. The data were recorded and averaged in 10-s sampling periods at 10-min intervals. Data acquisition and analysis were performed using Dataquest A.R.T. 4.3 software (Data Science International, St. Paul, Minnesota, USA).

A uniform protocol was used to record MAP and HR responses to biphalin in conscious SHR (n = 7), HS/UNX (n = 8) and Ang-iH rats (n = 7) and in normotensive Sprague-Dawley controls (n = 5), and the responses to morphine in Sprague-Dawley rats (n = 5). Thirty-min biphaline (300 µg h⁻¹kg⁻¹ BW) or morphine (1.5 mg h⁻¹kg⁻¹ BW) infusion was bracketed by 30-min pre-infusion (control) and post-infusion (recovery) periods. In each rat this protocol was repeated on five consecutive days and the data were averaged. These average values were used to calculate group means.

Statistics

A single comparison of mean values between the control and the experimental period was made using paired Student t-test. With multiple comparisons, the differences between means were first analysed using repeat measurement analysis of variance (ANOVA) followed by post-hoc modified t-tests, with Bonferroni correction (Wallenstein et al. 1980). The standard error of mean (SEM) was used as a measure of data dispersion and P ≤ 0.05 was accepted as the significance level.

RESULTS

Series I: Studies with anaesthetized rats

The main baseline haemodynamic characteristics of anaesthetized rats: UNX/HS, Ang-iH, normotensive S-D and SHR groups are collected in Table 1. Notably, in SHR the blood pressure was distinctly higher than in the two other models, at comparable HR values.
**Effects of biphalin.**

Since one major point of interest of the study was whether hypotensive action of biphalin would or would not be accompanied by a decrease in RBF, a review is first given of representative individual records of MAP and RBF responses in UNX/HS and Ang-iH rats, in a normotensive Sprague-Dawley rat, and in an SHR (Fig. 1). It is seen that the decrease in MAP was seen in SHR only: it started to develop within the first 2-3 minutes of infusion and reached an elevated plateau about five minutes later. This change was associated with an increase in RBF. In HS/UNIX and Sprague-Dawley rats increases in MAP were seen, they were also rapid in onset.

Full data on haemodynamic effects of biphalin in the HS/UNIX, Ang-iH and normotensive S-D groups are collected in Fig. 2 and the data on MAP and renal haemodynamics in SHR are given in Fig. 3. The crucial findings were about 6% MAP increases in HS/UNIX ($P<0.02$) and in normotensive S-D ($P<0.001$), contrasting with a 6% decrease in SHR ($P<0.001$), with no change in Ang-iH group. RBF increased 7% in HS/UNIX rats ($P<0.01$) without any change in RVR, apparently following the increase in MAP. On the other hand, the 10% RBF increase in SHR ($P<0.001$) (Fig. 3) was associated with a decrease in RVR. In normotensive S-D rats the decrease in RBF and the increase in RVR were significant but quantitatively negligible. In HS/UNIX, S-D and SHR rats after withdrawal of biphalin infusion, MAP and RBF promptly returned to values not different from pre-infusion controls. HR, RVR, and hind limb perfusion and vascular resistances (IBF, IVR) did not change significantly.

In Ang-iH rats MAP, HR, RBF and IBF were not altered by biphalin; the only change was a 12% decrease in hind limb vascular resistance (IVR) ($P<0.03$).

**Naloxone tests.**
In an additional group of seven normotensive SLD rats we showed that the post-biphalin systemic and renal haemodynamic changes were similar as in the main group shown in Fig. 2 (increases in MAP and RVR, and decreases in RBF and HR) and were not reversed by naloxone methiodide, with MAP of 121 ± 4 and 122 ± 3 mmHg, HR of 384 ± 14 and 396 ± 14 beats min\(^{-1}\), RBF of 9.3 ± 0.8 and 9.1 ± 0.8 ml min\(^{-1}\), and RVR of 13 ± 1 and 14 ± 1 mmHg(ml min\(^{-1}\))\(^{-1}\), under biphalin before and after naloxone, respectively (changes not significant).

In an additional group of six SHR we showed that the post-biphalin systemic and renal haemodynamic changes were similar as shown in Fig. 3 (decreases in MAP, HR, and RVR, and an increase in RBF). The responses were rapidly (within 3-5 min) reversed by naloxone methiodide, with MAP of 161 ± 7 and 173 ± 10 mmHg, HR of 336 ± 11 and 375 ± 6 beats min\(^{-1}\), RBF of 10.1 ± 0.6 and 9.0 ± 0.7 ml min\(^{-1}\), and RVR of 16 ± 2 and 20 ± 2 mmHg(ml min\(^{-1}\))\(^{-1}\), under biphalin before and after naloxone, respectively (differences significant at \(P<0.03-0.01\)).

**Effects of morphine.**

Compared to biphalin, the responses of anaesthetized hypertensive or normotensive rats to morphine were often delayed and progressed beyond the infusion time; evident recovery was unusual. Therefore the responses of MAP, HR, RBF, RVR, IBF and IVR to intravenous morphine (1.5 mg h\(^{-1}\)kg\(^{-1}\) BW) in hypertensive HS/UNX, Ang-iH and in normotensive S-D controls, are presented in a way to distinguish between two phases of the change during morphine infusion (Fig. 4).

In HS/UNX rats both MAP and RBF decreased, however, the decrease was clearly delayed, slowly progressed, and extended at least 20 min beyond morphine infusion time.
This was associated with a delayed increase in HR while IBF and renal and hind limb vascular resistances (RVR, IVR) remained unaltered.

A similar pattern of responses to morphine i.e. decreases in MAP, RBF and IBF was observed in normotensive S-D rats. Of note was a large decrease in MAP: from control of 121 ± 3 to 107 ± 5 mmHg in the second phase of infusion and further to 104 ± 4 mmHg after morphine withdrawal. This was a very substantial decrease (-14%, \(P<0.001\)), exceeding the MAP decrease in HS/UNX rats (-9%); such unexpected finding prompted us to check the response without anaesthesia (see Series II below).

In contrast to responses in HS/UNX and S-D, in Ang-iH rats MAP tended to increase in response to morphine infusion (NS), concurrent with a minor but significant decrease in HR (–3%). There was also a decrease in RBF and IBF, and significant increases in RVR and IVR (by about 27 and 11%, respectively) indicated peripheral vasoconstriction. The decrease in IBF and an increase in IVR progressed slowly, with no apparent recovery.

**Naloxone blockade of early effects of morphine in SHR.** We showed previously that in SHR the final effect of morphine (measured at the end of 20-25 min infusion and later after its withdrawal) consisted primarily in decreases in MAP and RBF and also of IVR; there was no change in RVR (Bądzyńska et al. 2016). Given the fluctuations and/or progressing character of morphine-induced changes, no attempt was then made to establish if the responses were naloxone-sensitive. In a group of SHR studied here (Table 2) we established that at least the initial post-morphine changes (decreases in MAP, HR (not significant), RVR and IVR measured after first 10 min of infusion) were reversed by naloxone. It should be noticed, however, that the initial RBF change, that reversed by naloxone, was, unlike the final effect described in our earlier study (Bądzyńska et al. 2016), a significant increase and not a decrease from control.
On the whole, the vasorelaxant and cardiodepressor action of morphine showed previously in SHR and WKY rats (Bądzyńska et al. 2016) was here largely confirmed in HS/UNX and normotensive S-D groups whereas Ang-iH rats proved resistant to such commonly recognized morphine action.

**Series II: Studies with conscious rats**

Effects of 30-min biphalin infusion on MAP and HR in conscious SHR, HS/UNX and Ang-iH rats are shown in Fig. 5, and those for normotensive S-D controls in the upper panel of Fig. 6.

In SHR group MAP promptly decreased during biphalin infusion, from pre-infusion control of 140 ± 4 it decreased and stabilized at 120 ± 3 mmHg during final 10 min of infusion (- 14%, *P*<0.001). After cessation of biphalin infusion a fairly rapid recovery was seen, to achieve the pre-infusion level at about 15 min after biphalin withdrawal. HR showed an almost parallel decrease (- 22%) except that, compared to MAP, the recovery was delayed by about 15 min. On the whole, a substantial and persistent decrease in MAP and HR was evident.

In HS/UNX group biphalin moderately but significantly decreased MAP by about 16 mmHg (- 12%), in parallel with a similar change in HR. However, beginning from about the 15th min of infusion a progressing recovery of both parameters started (despite continued infusion); by about 10 min after biphalin withdrawal MAP and HR have returned to control levels.

In Ang-iH group only some transient decreasing tendency for MAP and HR was seen, which disappeared despite continued biphalin infusion.

Fig. 6 (upper panel) shows that within the first 10 min of biphalin infusion in conscious normotensive S-D rats a MAP decrease by about 8 mmHg (- 8%, *P*<0.002) was seen, followed by progressing recovery to control level, despite continued infusion. A greater
decrease in HR (~20%) was somewhat delayed and so was the recovery which did not start until after 20 min of biphain infusion.

Fig. 6 (lower panel) shows that in conscious normotensive S-D rats morphine induced but a minor decrease in MAP (<4%). This was in contrast with a substantial (14%) decrease seen in anaesthetized rats (see above). Compared with a negligible decrease in MAP, there was a more substantial and significant decrease in HR (~10%). However, the effects were transient and both variables returned to control levels during morphine infusion, and after its withdrawal increased further and plateaued at a level significantly above control.

DISCUSSION

Biphain and morphine effects in SHR

The major finding of the present study is that biphain decreased blood pressure in SHR but not in the two other rat hypertension models (HS/UNX or Ang-iH), or in normotensive WKY and S-D rats. Jointly, our earlier results (Bądzyńska et al. 2016) and the present findings clearly show that hypotensive action of biphain is a highly reproducible phenomenon that can be demonstrated in SHR derived from different stocks, with different baseline MAP, studied at different seasons of the year, under or without anesthesia. A rapid onset of the hypotensive response to intravenous administration and rapid reversal of the response by blockade of peripheral opioid receptors suggest very strongly that stimulation of these receptors was the major mechanism of biphain’s effect.

We found that post-biphain MAP decreases in SHR, both under and without anaesthesia, were often associated with decreases in HR. This suggests that, in addition to a reduction of peripheral vascular resistance (RVR, IVR), a decrease in cardiac output could also contribute to the hypotensive response. However, in our earlier study in one of two groups of anaesthetized SHR the biphain-induced MAP decrease occurred even without any change in HR (Bądzyńska et al. 2016), and others reported that loperamide, another
peripherally acting μ-receptor agonist, did not alter the cardiac output in anesthetized SHR (Chen et al. 2011). Thus, any substantial contribution of a decrease in cardiac output to biphalin’s hypotensive effect remains uncertain.

As expected, also morphine, capable of stimulating both peripheral and central opioid receptors, induced a hypotensive response in SHR. The hypotensive action of morphine is long established (Cox 1988; Siren and Feuerstein 1992). More recently, both morphine and stimulation of peripheral μ receptors with loperamide was reported to decrease blood pressure in SHR (Chen et al. 2011, Mahinda et al. 2004). Also endogenous endorphins with affinity for μ receptors were reported to induce hind limb vasodilatation (Champion et al. 1997), in agreement with our present finding of decreasing iliac vascular resistance, both after morphine and after biphalin.

Earlier we confirmed the hypotensive effect of morphine (Bądzyńska et al. 2016) and here we showed that in the early phase morphine’s effects were mediated by peripheral receptors and were reversed by peripherally acting form of naloxone. However, compared to biphalin the full effects of morphine were usually delayed and most often extended beyond the time of infusion. This would be compatible with both weak peripheral action of morphine at the dose applied and not very rapid penetration of the BBB by the hydrophilic molecule, which retarded its action via central receptors. An alternative explanation of the delayed action of morphine could be the time required for its transformation to glucuronide derivatives, such as morphine-6-glucuronide which in the rat is distinctly more potent than the parent molecule (Osborne et al. 1992).

Neurogenic nature of hypertension in SHR and hypotensive effectiveness of biphalin

Given the established role of the renin-angiotensin (RAS) and sympathetic nervous (SNS) systems in control of arterial pressure and development of hypertension (Esler et al.
2010; Navar 2010; Schlaich et al. 2004), the known very complex interaction of opioids with these two regulatory systems (Bali et al. 2014; Barron 2000; Cox 1988; Siren and Feuerstein 1992,), and the fact that the SHR is a hypertension model with pronounced sympathetic hyperactivity (Lerman et al. 2005; Pinterova et al. 2011), the observation that hypotensive effect of biphinal was confined to this model provides some novel insight in the differences in pathogenesis of various forms of hypertension. The insight is further extended when the effects of biphinal, a peripherally active opioid, are compared with those of morphine which is known to exert both central and peripheral actions.

The SHR is long recognized to possess pronounced neurogenic background component, characterized primarily by increased sympathetic activity at different levels of the autonomous nervous system (Lerman et al. 2005; Pinterova et al. 2011). Remarkably, neonatal sympathectomy was reported to prevent the development of hypertension and of the structural vascular changes in SHR (Lee et al. 1987), and in SHR with established hypertension sympathetic activity was not effectively inhibited by baroreceptor activation dependent on elevation of blood pressure (Judy and Farrel 1979).

Therefore, hypotensive action of biphinal in SHR could be ascribed to peripheral opioid receptor-mediated reduction of adrenergic tone; the expected result would be vasorelaxation and perhaps also cardiodepression. At peripheral sites, opioid peptides are known to suppress the autonomic nerve activity by inhibiting the presynaptic release of neurotransmitters but also exert postsynaptic actions (Barron 2000; Cox 1988); they can also decrease the sensitivity of the vessel wall to adrenergic agents (Champion et al. 1997).

It can be expected that the peripheral sympathoinhibitory action of opioids, also the hypotensive effect, would be more apparent when baseline sympathetic neural input to the vasculature and/or vascular responsiveness to adrenergic stimuli are elevated, as in SHR. On
the other hand, biphadin showed no hypotensive effects in normotensive WKY rats (Bądzyńska et al. 2016) and, as shown in this study, in normotensive S-D rats, the strains with presumably normal SNS activity.

**Why no hypotensive action of biphadin in two non-SHR models of hypertension?**

Considering that (1) high baseline SNS activity is probably prerequisite for the hypotensive response to biphadin, (2) there is a close interrelationship of RAS and SNS, and (3) there are very complex interactions of brain RAS and opioids (Bali et al. 2014), different baseline SNS and RAS status in the hypertension models used may have determined the differential vascular and MAP responses.

**Role of baseline SNS status.** Whether there is an important sympathoexcitation in angiotensin-induced hypertension remains a matter of debate (Lohmeier 2012). Stimulation of brain RAS is known to increase sympathetic output from cardiovascular regulatory centres (Pinterova et al. 2011). However, it is not very likely that at subpressor dosage Ang II would penetrate BBB in quantities sufficient to stimulate sympathetic output from brain centres. Norepinephrine turnover measurements in the heart, kidney, skeletal muscle and intestine did not support the hypothesis that rats with angiotensin-induced hypertension have enhanced sympathetic tone (Kline et al. 1990). There is evidence that sympathoexcitation would occur as a result of Ang II treatment only when combined with high salt intake (Guild et al. 2012; King et al. 2008; Osborn et al. 2007) but more recently even such combination did not result in elevation of sympathetic nerve activity in the kidney and hind limb vascular beds (Yoshimoto et al. 2010). Therefore, it is unlikely that baseline sympathoexcitation was an important feature in our Ang-iH rats maintained on standard diet, which would be compatible with the absence of a hypotensive response to biphadin.

In high salt intake hypertension plasma RAS activity is suppressed but that in the brain...
may be elevated, leading to increasing local oxidative stress (Kishi and Hirooka 2012), central sympathoexcitation, and enhanced neural traffic to organs and tissues. This was clearly shown in Dahl salt sensitive rats (Zhao et al. 2001). However, in young Sprague-Dawley rats with hypertension induced by uninephrectomy followed by 4 weeks’ high salt intake, sympathoexcitation was also suggested on basis of increased MAP response to ganglionic blockade (Fujita et al. 2012). Therefore, our HS/UNX rats could also display sympathetic hyperactivity, similarly as the SHR model, however, no hypotensive response to biphain was seen.

It will be noticed, however, that peripherally acting biphalin would oppose adrenergic hyperactivity mostly at the periphery and would be less likely to affect sympathetic output from brain centres. The feature crucial for hypotensive response to peripheral opioid stimulation in SHR might be increased responsiveness of peripheral vessels to adrenergic stimuli. Indeed, exaggerated increase in total peripheral vascular resistance was seen in SHR after experimentally induced release of norepinephrine (Berg 2005). More important, opioids were shown to inhibit this hypersensitivity: vasoconstrictor response to electrical stimulation of rat tail artery was blocked by an opioid µ-receptor agonist in arteries derived from SHR but not those from normotensive rats (Wong and Ingenito 1995). Furthermore, stimulation of peripheral µ receptors was reported to decrease blood pressure more effectively in SHR than in normotensive rats (Mahinda et al. 2004) or to do so exclusively in SHR (Chen et al. 2011).

No evidence is available for enhanced vascular responsiveness to adrenergic stimulation in the HS/UNX or similar models. Therefore we propose that inhibition by biphalin of the elevated responsiveness of the blood vessel wall to adrenergic stimulation was crucial for the vasorelaxation and hypotensive effect in SHR.
**Role of baseline RAS status.** It is commonly accepted that Ang II facilitates sympathetic transmission to the vascular wall (Pinterova et al. 2011) and opioid peptides suppress neurotransmitter release (Barron 2000; Cox 1988) and can also decrease the sensitivity of the vasculature to adrenergic agents (Champion et al. 1997). In the light of extensive studies and review of the literature (Campbell et al. 1995), plasma and tissue renin or Ang II levels are not elevated in adult SHR, and high salt intake, as in HS/UNX rats, suppresses plasma RAS activity. Therefore, it's unlikely that in these two models the RAS status determined the nature of the vascular response to opioids. However, this possibility should be considered for the Ang-iH model, even though with subpressor Ang II dosage the brain peptide elevation sufficient to increase sympathetic output and thereby alter the vascular tone seems unlikely. Beside modest elevation of plasma peptide level, there is a distinct increase in intrarenal Ang II activity, due both to an uptake from plasma and stimulation of intrarenal synthesis (Zou et al. 1996).

Thus, peripherally acting biphinalin could antagonize exaggerated vasoconstrictor influence of increased adrenergic tone induced by Ang II present in plasma and tissues. However, no hypotensive response was actually seen. One reason could be the well known angiotensin-dependent functional and structural remodelling of the vessel wall (Heeneman et al. 2007; Montezano et al. 2014), which could render it unresponsive to opioid-mediated signalling. This could explain the inability of resistance vessels of Ang-iH rats to dilate. Remarkably, we found that also morphine failed to decrease vascular resistances and MAP in Ang-iH rats, in contrast to the two other models of hypertension.

It is obvious that interpretation of the biphalin’s hypotensive effect which proved selective for SHR cannot be solely sought in the animals’ functional status of SNS and RAS. The role of other regulatory and mediating systems, e.g. the degree and alterations of the brain and systemic (especially in the vascular endothelium) oxidative stress and, perhaps more
important, the activity status of nitric oxide might be involved. Morphine and many opioid analgesics are known to release histamine (Baldo and Pham 2012) and its mediatory role should also be considered. Appropriately focused studies would be needed to address the contribution of these systems and active agents.

**Paradoxical increases in MAP after biphalin**

Remains the question why in normotensive S-D and in HS/UNIX rats biphalin caused a modest increase in MAP, be it only under anaesthesia. Remarkably, another enkephalin analogue, [Leu⁵]enkephalin was also reported to cause vasoconstriction and blood pressure increase (Crooks et al. 1984; Giles and Sander 1983). While opioids can directly stimulate brain centres leading to enhancement of sympathetic output and to peripheral vasoconstriction (Bali et al. 2014; Cox 1988, Siren and Feuerstein 1992), the rapid onset of biphalin’s action suggested the mediatory role of peripheral receptors. However, such rapid pressure increase could occur in case of stimulation by opioids of brain centres devoid of BBB, such as circumventricular organs, especially the area postrema (AP). There is evidence that AP is the site of Ang II dependent sympathoexcitation (Szilagyi and Ferrario 1981) and AP ablation was found to prevent the maintenance of chronic angiotensin-induced hypertension (Fink et al 1987). It is totally unclear why of the two normotensive strains the post-biphalin blood pressure increase, even if only under anaesthesia, was seen in S-D but not, as reported earlier, in WKY rats (Bądzyńska et al. 2016). Regarding HS/UNIX rats, it could be speculated that hypertension induced damage of BBB could accelerate biphalin penetration also to brain sites other than AP, which could induce peripheral vasoconstriction.

Since in both S-D and HS/UNIX rats a clear hypotensive response was seen after morphine, one could also suspect that the unusual response to biphalin was nonspecific, perhaps unrelated to stimulation of opioid receptors. Indeed, we found that in our normotensive S-D rats the increase in blood pressure, at least in the early phase of biphalin’s
action, was not reversed by naloxone. Nor was such reversal seen in the case of vasoconstrictor action of [Leu⁵]enkephalin (Crooks et al. 1984).

**Biphalin versus morphine effects on RBF and potential application of biphalin-like drugs in antihypertensive therapy**

We confirmed here the unexpected earlier finding that in SHR biphalin increased RBF and lowered RVR despite a decrease in MAP (Bądżyńska et al. 2016). This indicates a specific vasodilator response of the renal vasculature that might be important if application of similar opioids in antihypertensive therapy were considered.

Similarly as in the case of MAP, morphine effect on RBF was biphasic: there was an initial significant increase associated with a fall in RVR, the changes which were reversed by peripherally acting form of naloxone (Table 2). This phase must have reflected prompt stimulation of peripheral opioid receptors, an effect that was later prevailed upon by stimulation of central receptors, which ultimately caused a decrease in RBF, as described previously (Bądżyńska et al. 2016). This suggests that renal vasorelaxation observed with biphalin and in the first phase of morphine’s action was due to stimulation confined to peripheral opioid receptors located in the renal vasculature.

Biphalin or other synthetic opioid receptor agonists with pronounced peripheral action, originally designed to provide non-addictive analgesia, could be of value in the treatment of hypertension forms with pronounced sympathetic overactivity, especially in essential hypertension. Of notice is biphalin’s beneficial action in the SHR, the model which in many respects resembles essential hypertension in humans (Schlaich et al. 2004); application in states of hypertension directly related to physical and/or mental stress could also be considered. The drug’s important advantages are that in contrast to morphine it is non-addictive and does not impair renal perfusion.
In conclusion, the major novel finding of this study was that the hypotensive action of intravenous biphalin, a synthetic enkephalin analogue, was a phenomenon demonstrable in both anaesthetized and conscious SHR but not in two other essentially different models of rat hypertension or in normotensive Sprague-Dawley controls. Such selective hypotensive effect could occur because of the established characteristics of SHR: increased baseline sympathetic traffic to resistance vessels and their increased responsiveness to sympathetic stimulation. Biphalin’s hypotensive action was primarily due to peripheral vasorelaxation; despite a decrease in blood pressure, perfusion of the kidney was not impaired.

The other major finding was that rats with angiotensin-induced hypertension were resistant to vasomotor influence of both biphalin and morphine, most probably due to angiotensin induced functional and structural remodelling of the resistance vessel wall. Our findings indicate that success or failure of an antihypertensive treatment regime may critically depend on the pathogenesis of hypertension. An advantage of biphalin-like drugs, should they ever be applied in therapy, would be that their application for antihypertensive treatment would not endanger renal perfusion.
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Competing Interests

All authors declare that there are no competing financial, personal or professional interests.

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Table 1. Baseline values for mean arterial pressure (MAP), heart rate (HR), renal vascular resistance (RVR) and iliac (i.e. hind limb) vascular resistance (IVR) in three rat models of hypertension and in normotensive S-D rats.

<table>
<thead>
<tr>
<th>Model</th>
<th>n</th>
<th>MAP (mmHg)</th>
<th>HR (beats min⁻¹)</th>
<th>RVR (mmHg (ml min⁻¹)⁻¹)</th>
<th>IVR</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS/UNX</td>
<td>20</td>
<td>150 ± 3</td>
<td>368 ± 9</td>
<td>12 ± 1</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>Ang-iH</td>
<td>19</td>
<td>145 ± 3</td>
<td>396 ± 8</td>
<td>15 ± 1</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>S-D</td>
<td>15</td>
<td>119 ± 1</td>
<td>397 ± 6</td>
<td>12 ± 1</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>SHR</td>
<td>8</td>
<td>170 ± 5</td>
<td>392 ± 4</td>
<td>16 ± 1</td>
<td>-</td>
</tr>
</tbody>
</table>

Means ± SEM. HS/UNX – unilaterally nephrectomized rats on high-sodium diet; Ang-iH – angiotensin-induced hypertension; SHR – spontaneously hypertensive rats. For HS/UNX and Ang-iH groups pooled data for rats later receiving biphalin or morphine are shown. For IVR in the S-D group n = 5.
Table 2. Reversal by naloxone of the early effect of morphine on MAP and renal and hind limb perfusion parameters in SHR.

<table>
<thead>
<tr>
<th></th>
<th>SHR ( n = 11 )</th>
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<tbody>
<tr>
<td></td>
<td>control</td>
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<tr>
<td>MAP mmHg</td>
<td>155 ± 3</td>
</tr>
<tr>
<td>HR beats min(^{-1})</td>
<td>346 ± 14</td>
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<tr>
<td>RBF ml min(^{-1})</td>
<td>8.5 ± 0.4</td>
</tr>
<tr>
<td>RVR mmHg (ml min(^{-1}))(^{-1})</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>IBF ml min(^{-1})</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>IVR mmHg (ml min(^{-1}))(^{-1})</td>
<td>50 ± 4</td>
</tr>
</tbody>
</table>

Means ± SEM. Drug dosage: morphine - 1.5 mg kg\(^{-1}\)h\(^{-1}\), naloxone - 200 µg kg\(^{-1}\). RBF – renal blood flow, IBF – iliac blood flow. Other denotations as in Tab. 1. * significantly different from the preceding value, by repeat measurement ANOVA followed by modified paired t-test.
Figure legends

Figure 1. Individual records of MAP and RBF responses to biphalin (300 µg h\(^{-1}\)kg\(^{-1}\) BW) in anaesthetized rats representing UNX/HS and Ang-iH, in a control Sprague-Dawley rat, and in an SHR.

The data expressed as changes from 100% baseline value. MAP and RBF denote mean arterial pressure and left kidney blood flow, respectively. HS/UNX: hypertension induced in Sprague-Dawley (S-D) rats by uninephrectomy followed by high salt intake; Ang-iH: angiotensin-induced hypertension in S-D rats.

Figure 2. The responses of MAP, HR, RBF, RVR, IBF and IVR to intravenous biphalin (300 µg h\(^{-1}\)kg\(^{-1}\) BW) in anaesthetized rats: hypertensive HS/UNX (n = 11) and Ang-iH rats (n = 8), and in normotensive S-D controls (n = 12-15; for IBF and IVR n = 5). Means ± SEM. HR, heart rate; RVR, renal vascular resistance; IBF, iliac (hind limb) blood flow; IVR, iliac vascular resistance. Other denotations as in Fig. 1. *significantly different from control by paired t-test.

Figure 3. MAP, HR, RBF and RVR responses to intravenous biphalin (300 µg h\(^{-1}\)kg\(^{-1}\) BW) in eight anaesthetized spontaneously hypertensive rats (SHR)

Means ± SEM. Denotations as in Figs. 1 and 2.

Figure 4. The responses of MAP, HR, RBF, RVR, IBF and IVR to intravenous morphine (1.5 mg h\(^{-1}\)kg\(^{-1}\) BW) in anaesthetized rats: hypertensive HS/UNX (n = 10) and Ang-iH rats (n = 11), and in normotensive S-D controls (n = 12).

Blank bars represent saline control values, the first black bar - the value recorded at the 10\(^{th}\) min of morphine infusion, the second black bar – the value averaged for the final (25-30 min) of morphine infusion, and striped bars – the value recorded 15-25 min after cessation of morphine infusion (compare Fig. 3). Means ± SEM. Denotations as in Fig. 1 and 2.
*significantly different from control by repeat measurement ANOVA followed by modified t-test, with Bonferroni correction.

Figure 5. MAP and HR responses to intravenous biphalin (300 µg h\(^{-1}\)kg\(^{-1}\) BW) in conscious rats of three hypertension models: SHR, UNX/HS and Ang-iH. Means ± SEM. Dotted lines and open symbols refer to infusions of saline solvent. Denotations as in Fig. 1 and 2. *significantly different from control by repeat measurement ANOVA followed by modified t-test.

Figure 6. MAP and HR responses to intravenous to biphalin, 300 µg h\(^{-1}\)kg\(^{-1}\) BW (upper panel, \(n =5\)) and to intravenous morphine, 1.5 mg h\(^{-1}\)kg\(^{-1}\) BW (lower panel, \(n =5\)) in conscious normotensive S-D rats. Means ± SEM. Dotted lines and open symbols refer to infusions of saline solvent. Denotations as in Fig. 1 and 2. *significantly different from control by repeat measurement ANOVA followed by modified t-test.
ANAESTHETIZED RATS

Figure 1
Figure 2

ANAESTHETIZED RATS

MAP

HR

RBF

IBF

RVR

IVR

**control**  **biphalin**  **recovery**

<table>
<thead>
<tr>
<th>mmHg</th>
<th>control</th>
<th>biphalin</th>
<th>recovery</th>
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<tr>
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<tr>
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<table>
<thead>
<tr>
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<tr>
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<table>
<thead>
<tr>
<th>RBF</th>
<th>ml min⁻¹</th>
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<td>50</td>
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<table>
<thead>
<tr>
<th>RVR</th>
<th>mmHg (ml min⁻¹)⁻¹</th>
<th>IVR</th>
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<td>40</td>
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<table>
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<th>Ang-iH</th>
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</table>

Figure 2

https://mc06.manuscriptcentral.com/cjpp-pubs
Figure 3

ANAESTHETIZED SHR

MAP

mmHg

120 130 140 150 160 170 180

120 130 140 150 160 170 180

beats min⁻¹

200 250 300 350 400 450

200 250 300 350 400 450

RBF

ml min⁻¹

0 2 4 6 8 10 12

0 2 4 6 8 10 12

control biphainl recovery

RVR

mmHg/ml min⁻¹

0 5 10 15 20

0 5 10 15 20

*
ANAESTHETIZED RATS

Figure 4
Figure 5

CONSCIOUS RATS

**MAP**

**SHR**

**HR**

**MAP**

**HS/UNX**

**HR**

**MAP**

**Ang-iH**

**HR**

**MAP**

**Ang-iH**

**HR**

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CONSCIOUS SPRAQUE-DAWLEY RATS

**Figure 6**

MAP

**BIPHALIN**

HR

**MORPHINE**

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