Prolonged Inhibition of Neutral Endopeptidase 24.11 by Sinorphan in Stroke-Prone Spontaneously Hypertensive Rats

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The cardiovascular consequences of inhibition of the neutral endopeptidase 24.11 (NEP) with the orally active NEP inhibitor sinorphan were evaluated by determining long-term effects of the drug on hemodynamic, hormonal and structural parameters in stroke-prone spontaneously hypertensive rats (SHR-SP). Systolic blood pressure increased in young SHR-SP from 194 ± 2 to 266 ± 7 mmHg, whereas in sinorphan (30 mg/kg p.o. bid) treated animals systolic blood pressure increased only from 193 ± 4 to 229 ± 4 mmHg during the treatment period of 9 weeks. The increase in relative heart weight was also delayed. Plasma ANP was higher in the sinorphan group than in the controls. The results of a second study demonstrate a substantial improvement of cardiac pump function and ventricular hypertrophy in old SHR-SP with compromised cardiac function by long-term inhibition of NEP. Thirteen-month-old SHR-SP were treated with sinorphan (30 mg/kg p.o. bid) for two weeks. At the end of experiment, the increase in ANP plasma levels did not reach statistical significance, whereas plasma cGMP was higher in sinorphan treated animals than in controls. Left ventricular end-diastolic pressure was markedly elevated in controls and significantly lower in sinorphan treated animals. In addition, sinorphan reduced cardiac hypertrophy in these old SHR-SP. In conclusion, the results of the present studies demonstrate that long-term NEP inhibition with sinorphan has inhibitory effects on malignant hypertension and associated cardiac hypertrophy in young SHR-SP on a high-sodium diet. NEP inhibition substantially improves cardiac pump function and reduces ventricular hypertrophy of old SHR-SP with compromised cardiac function. (*Hypertens Res* 1995; 18: 137-143)

Key Words: atrial natriuretic peptide, neutral endopeptidase inhibitor, stroke prone spontaneously hypertensive rat, cardiac hypertrophy, congestive heart failure

Atrial natriuretic peptides (ANP), which are secreted by atrial myocytes in response to increased intravascular volume and atrial stretch, have potent natriuretic, diuretic, and vasorelaxant properties and suppress the renin-angiotensin-aldosterone system. ANP is cleared from plasma by ANP clearance receptors (ANP C receptors) and by enzymatic degradation by neutral endopeptidase 24.11 (NEP). It has been shown that inhibition of the NEP causes a blood pressure decrease in response to increased ANP plasma levels (1-4). Obviously, these interesting actions of ANP have led many investigators to speculate that NEP inhibitors could be valuable therapeutic tools for the treatment of disorders such as hypertension, renal failure and congestive heart failure (4-8). Indeed, most studies using NEP inhibitors have confirmed significant increases in plasma ANP (9-14); a few have not (15-17). However, data on the depressor effects of NEP inhibitors are conflicting. NEP inhibitors do not decrease blood pressure in animals with congestive heart failure (10-12) or in those with chronic renal failure. With

regard to hypertensive animals, NEP inhibition substantially decreased blood pressure in DOCA/salt rats (14-17) and in Dahl salt-sensitive rats, but not in spontaneously hypertensive rats (SHR) in acute experiments (13, 15, 16, 18-21).

Arterial hypertension is a major risk factor for damage of the brain, kidney and heart in humans. Spontaneously hypertensive rats and their stroke-prone substrain (SHR-SP) have been employed widely as a model of human hypertensive disease. SHR-SP develop malignant hypertension accompanied by extensive end-organ damage, including malignant nephrosclerosis, cerebrovascular lesions, and brain infarctions (22-24). Dietary salt load in young SHR-SP has been shown to intensify hypertension and renal and cerebrovascular lesions (23-25). SHR-SP initially fail to suppress and later paradoxically increase renin secretion when fed a high-sodium diet (25, 26). Morbidity and mortality are increased due to renal and cerebrovascular lesions in these rats and are associated with a rise in activity of the renin-angiotensin system (25, 26).

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Little is known with regard to the effects of chronic NEP inhibition in stroke-prone spontaneously hypertensive rats (SHR-SP). Here, we report the results of a preventive trial with the NEP inhibitor sinorphan (4-8, 29), which was administered orally to young SHR-SP on a high-salt diet (8% NaCl) for 9 weeks. In a second experiment, sinorphan was administered for 2 weeks to aged SHR-SP with congestive heart failure. The aim of this study was to evaluate the cardiovascular consequences of inhibition of the neutral endopeptidase 24.11 (NEP) with the orally active NEP inhibitor sinorphan by determining long-term effects of the drug on hemodynamics, and on hormonal and structural parameters in SHR-SP.

Material and Methods

Animals and Treatment

Forty SHR-SP were obtained from the Central Animal Facilities of Bayer AG. They were housed under controlled conditions of light and temperature.

Study 1: At the age of 10 weeks, the animals were fed a high salt diet (8% NaCl in a commercial diet, Ssniff Versuchstierdiäten, Soest, Germany) and were treated with 30 mg/kg sinorphan orally by gavage at 8:00 a.m. and 4:00 p.m. for 9 weeks. Sinorphan was administered as a suspension in polyethylene glycol 400/carboxymethyl cellulose (0.5%) solution (v/v=10/90). The administration volume was 2 ml/kg body weight. The controls received the vehicle. Systolic blood pressure was measured every week by the tail-cuff method in conscious animals, prewarmed in thermostatic cages at 37°C. At the end of the study, all animals were weighed and killed by decapitation. Blood samples were collected after decapitation into prechilled EDTA tubes.

Study 2: Fifty-six-week-old male SHR-SP were used in this experiment and fed standard rat chow containing 0.4% NaCl. The blood pressure of these animals had already passed its summit and was slowly declining. Systolic blood pressures were in the range of 180 mmHg. At the age of 56 weeks, more than 50% of the initial group of animals had already died. The animals were treated with 30 mg/kg sinorphan orally by gavage at 8:00 a.m. and 4:00 p.m. The controls received vehicle only. After 14 days of treatment, animals were anesthetized with 3% halothane in air. After completion of the hemodynamic measurements, the animals were bled from a carotid artery catheter. Blood samples were collected into prechilled EDTA tubes.

After thoracotomy, the kidneys and the hearts were removed and the ventricles isolated by cutting off the atria, pulmonary arteries, and aortas. The ventricles were opened, washed, blotted dry with filter paper, and weighed.

Plasma Determinations

Plasma renin activity (PRA) was determined by incubation of rat EDTA plasma with phenylmethylsulfonyl fluoride. The accumulated angiotensin I was measured with a commercially available

radioimmunoassay kit (Sorin Biomedica, Saluggia, Italy).

For determination of cyclic GMP in plasma, an equal volume of ice-cold 10% trichloroacetic acid was added to the samples at 4°C. After centrifugation the supernatant was extracted four times with water-saturated ether to remove the acid, lyophilized, redissolved in sodium acetate buffer, and assayed with a commercially available radioimmunoassay kit (IBL, Hamburg, Germany).

ANP in plasma was measured after extraction with C₁₈-cartridges by a specific radioimmunoassay as previously described (28).

Plasma creatinine was determined by a spectrometric method (29), and plasma urea was determined by an enzymatic UV test (30).

Renal Parameters

In the last week of experiments, the rats were placed into metabolic cages for determination of renal excretion. Urinary sodium and potassium were determined by flame photometry (Laboratory Instruments, USA). Cyclic GMP in urine was measured with a commercially available radioimmunoassay kit (IBL, Hamburg, Germany).

Hemodynamics

For the measurement of left ventricular pressure, a micro-tip blood pressure catheter (Millar SPR-249) was inserted into the right carotid artery and advanced into the left ventricle. After placement of the catheter, a steady-state concentration of 0.8% halothane was applied for exactly 10 min, and at least 10 cardiac cycles were registered at 250 mm/s paper speed. Left ventricular pressure and its first derivative (dp/dt) were recorded simultaneously. End-diastolic pressure was taken at the point of time when the first derivative of left ventricular pressure began to rise above baseline. Each individual value is the mean of 10 cardiac cycles. After registration the catheter was removed and its baseline drift was recorded for correction of end-diastolic pressure. Control and treated animals were studied in a random order. Typical rates of rise of left ventricular pressure and end-diastolic pressure obtained in normotensive aged Wistar rats of 440 g body weight were $6,231 \pm 256$ mmHg/s and 3.3 ± 0.5 mmHg ($n=17$), respectively.

Statistics

Differences between means were analyzed by Student's *t*-test after testing samples for normal distribution. Significant differences were assumed when $p < 0.05$. Results are presented as means $\pm$ SEM.

Results

Study 1

The development of systolic blood pressure in 10-week-old SHR-SP on a high sodium diet could be delayed significantly by sinorphan treatment (30 mg/kg p.o. bid). Systolic blood pressure increased in control animals from 194 ± 2 mmHg to 266 ± 7 mmHg during the observation period of 9 weeks

Fig. 1. Development of systolic blood pressure in sinorphan treated and untreated SHR-SP fed a high salt diet (8% NaCl) for 9 weeks. Number over or under the curve shows number of surviving animals. Values are means $\pm$ SEM. * $p < 0.05$, *** $p < 0.005$ compared with values in untreated controls.

Fig. 2. Development of body weight in sinorphan treated and untreated SHR-SP fed a high salt diet (8% NaCl) for 9 weeks. Number over or under the curve shows number of surviving animals. Values are means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared with values in untreated controls.

Table 1. Study 1: The Effect of Long-Term Treatment (9 wk) with Sinorphan (30 mg/kg p.o. bid) in Young SHR-SP on 8% NaCl on Blood Pressure, Body Weight (BW), Heart Weight, Kidney Weight, and Mortality

Variable	Control	Sinorphan
Blood pressure (mmHg)	266 $\pm$ 6.7 (13)	229 $\pm$ 4.2 (16) [#]
Body weight (g)	201 $\pm$ 9.4 (13)	233 $\pm$ 5.5 (16) ^{**}
Relative heart weight (mg/100 g BW)	475 $\pm$ 14 (13)	422 $\pm$ 4.9 (16) ^{***}
Relative kidney weight (mg/100 g BW)	1,061 $\pm$ 49 (13)	921 $\pm$ 20 (16) [*]
Mortality	7/20	4/20

Values are means $\pm$ SEM (n). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, [#] $p < 0.001$ compared with values in untreated controls.

(Fig. 1). In the sinorphan treated animals, systolic blood pressure increased from 193 ± 4 mmHg to 229 ± 4 mmHg during the treatment period. In contrast, sinorphan did not decrease systolic blood pressure significantly in 10-week-old SHR-SP and 19-week-old salt-loaded SHR-SP given 10, 30 and 100 mg/kg p.o. in single-dose experiments (data not shown).

After 9 weeks, 80% of the rats receiving sinorphan, but only 65% of the controls, were still alive (Fig. 1, Table 1). Higher morbidity in controls than in sinorphan treated rats was also suggested by body weight. Following 5 weeks of a high salt diet, untreated rats during the last 5 weeks of the experiment lost body weight, whereas the sinorphan treated rats did not (Fig. 2, Table 1). Relative heart weights and relative kidney weights were significantly lower in the sinorphan group (Table 1).

Table 2 summarizes basic plasma variables in the controls and sinorphan treated rats at the end of the 9-week experimental period. ANP plasma levels increased significantly during sinorphan treatment.

Although statistical analysis did not show any significant differences, the plasma renin activity and plasma aldosterone concentration tended to be higher in the control SHR-SP group than in the sinorphan group. Plasma urea was significantly lower and hematocrit significantly higher in the sinorphan group. Plasma creatinine and plasma sodium were comparable in the two groups, as shown in Table 2.

In the 9th week of treating the SHR-SP on a high salt diet with sinorphan, indices of renal function were measured. No significant effects were observed with respect to diuresis, natriuresis and kaliuresis, whereas cyclic GMP excretion was significantly elevated (Table 3).

Study 2

After 14 days of sinorphan treatment of old SHR-SP with end-stage hypertension, plasma ANP tended to increase, but no statistically significant difference was found (Table 4). Similarly, no significant differences in plasma angiotensin I and plasma

Table 2. Study 1: The Effect of Long-Term Treatment (9 wk) with Sinorphan (30 mg/kg p.o. bid) in Young SHR-SP on 8% NaCl on Atrial Natriuretic Peptides (ANP), Renin Activity (PRA), Angiotensin I, Aldosterone, Urea, Sodium and Creatinine in Plasma and Hematocrit

Variable	Control	Sinorphan
ANP (pg/ml)	265 ± 22 (10)	455 ± 25 (16) [#]
PRA (ng/ml/h)	15.3 ± 2.6 (13)	13.2 ± 1.6 (16)
Angiotensin I (ng/ml)	2.3 ± 0.3 (13)	2.2 ± 0.3 (16)
Aldosterone (pg/ml)	128 ± 26 (13)	94 ± 26 (14)
Urea plasma (mmol/l)	9.2 ± 1.0 (13)	6.9 ± 0.2 (16) [*]
Sodium (mmol/l)	132 ± 1.0 (13)	133 ± 0.7 (16)
Creatinine (μmol/l)	45 ± 2.0 (13)	45 ± 1.1 (16)
Hematocrit (%)	35 ± 1.5 (13)	39 ± 0.9 (16) [*]

Values are means ± SEM (*n*). ^{*}*p* < 0.05, [#]*p* < 0.001 compared with values in untreated controls.

Table 3. Study 1: The Effect of Long-Term Treatment (9 wk) with Sinorphan (30 mg/kg p.o. bid) in Young SHR-SP on 8% NaCl on Urine Volume, Sodium, Potassium, and Cyclic GMP Excretion. Collecting Period 6 h

Variable	Control	Sinorphan
Diuresis (ml/kg/h)	11.8 ± 0.9 (13)	12.8 ± 0.8 (16)
Natriuresis (μmol/kg/h)	1,878 ± 150 (13)	2,046 ± 144 (16)
Kaliuresis (μmol/kg/h)	330 ± 24 (13)	372 ± 30 (16)
cGMP excretion (nmol/kg/h)	4.2 ± 0.9 (13)	9.3 ± 1.5 (16) ^{**}

Values are means ± SEM (*n*). ^{**}*p* < 0.01 compared with values in untreated controls.

Table 4. Study 2: Influence of 14 Days of Treatment with Sinorphan (30 mg/kg p.o. bid) in Old SHR-SP on ANP, cGMP, Angiotensin I, Renin Activity (PRA) in Plasma Body Weight (BW), Relative Heart Weight, and Relative Kidney Weight

Variable	Control	Sinorphan
ANP (pg/ml)	471 ± 67 (11)	612 ± 122 (11)
cGMP (pmol/ml)	27.2 ± 4.6 (11)	41.8 ± 4.8 (11) ^{***}
Angiotensin I (ng/ml)	2.8 ± 0.6 (11)	2.1 ± 0.3 (11)
PRA (ng/ml/h)	11.7 ± 3.3 (11)	9.2 ± 0.9 (11)
Body weight (g)	332 ± 3 (12)	334 ± 5 (12)
Relative heart weight (mg/100 g BW)	460 ± 14 (12)	420 ± 6 (12) [*]
Relative kidney weight (mg/100 g BW)	802 ± 16 (12)	801 ± 16 (12)

Values are means ± SEM (*n*). ^{*}*p* < 0.05, ^{***}*p* < 0.005 compared with values in untreated controls.

Table 5. Study 2: Influence of 14 Days of Treatment with Sinorphan (30 mg/kg p.o. bid) in Old SHR-SP on Left Ventricular (LV) Rate of Rise, LV Systolic Pressure, LV End-Diastolic Pressure, and Heart Rate

Variable	Control	Sinorphan
LV rate of rise (mmHg/s)	6,880 ± 209 (12)	7,431 ± 226 (11)
LV systolic pressure (mmHg)	171 ± 4 (12)	166 ± 5 (11)
LV end-diastolic pressure (mmHg)	24.3 ± 0.7 (12)	15.9 ± 2.0 (11) [#]
Heart rate (beats/min)	312 ± 8 (12)	297 ± 9 (11)

Values are means ± SEM (*n*). [#]*p* < 0.001 compared with values in untreated controls.

renin activity were noted, although values tended to be lower in sinorphan treated rats (Table 4). However, cGMP in plasma, as a marker for ANP involvement, was increased in animals treated with sinorphan.

Heart rate, left ventricular systolic pressure, and left ventricular rate of rise in systolic pressure were

unaffected by sinorphan (Table 5). Left ventricular end-diastolic pressure, however, was markedly elevated in control animals, exceeding 20 mmHg (Table 5). Young normotensive Wistar rats reach values of 4 mmHg under the experimental conditions used (data not shown).

Aged SHR-SP treated with sinorphan had signif-

Table 6. Study 1: Influence of 14 Days of Treatment with Sinorphan (30 mg/kg p.o. bid) in Old SHR-SP on Urine Volume, Sodium, Potassium, and Cyclic GMP Excretion. Collecting Period 24 h

Variable	Control	Sinorphan
Diuresis (ml/kg/h)	2.8±0.4 (12)	2.5±0.2 (12)
Natriuresis (μmol/kg/h)	230±16 (12)	249±12 (12)
Kaliuresis (μmol/kg/h)	341±20 (12)	277±16 (12)*
cGMP excretion (nmol/kg/h)	2.2±0.3 (12)	5.4±0.6 (12)#

Values are means±SEM (*n*). **p*<0.05, #*p*<0.001 compared with values in untreated controls.

icantly reduced end-diastolic pressures (Table 5). In addition, treatment with sinorphan also reduced the increased heart weights of these rats (Table 4).

In the second week of treating the old SHR-SP with sinorphan, indices of renal function were measured. No significant effects were observed with respect to diuresis and natriuresis, whereas kaliuresis and cyclic GMP excretion was significantly elevated (Table 6).

Discussion

The cardiovascular consequences of NEP inhibition with sinorphan were evaluated by determining long-term effects of the drug on hemodynamic, hormonal, and structural variables in two experimental animal models of hypertension: young SHR-SP on a high-sodium diet and aged SHR-SP with congestive heart failure. SHR-SP develop malignant hypertension accompanied by extensive end-organ damage, including malignant nephrosclerosis, cerebrovascular lesions, and brain infarctions (22–24). Dietary salt load in young SHR-SP has been shown to intensify hypertension and renal and cerebrovascular lesions (23–25). Morbidity and mortality increased due to renal and cerebrovascular lesions in these rats, associated with a rise in the activity of the renin-angiotensin system (25, 26). Plasma levels of ANP in SHR-SP are elevated and may reflect a compensatory response of the rat to maintain normal blood pressure and plasma volume. We surmised that sinorphan, by preventing degradation of ANP, would potentiate the activity of the endogenous hormone and thereby prevent the development of hypertension and associated cardiac hypertrophy. The development of systolic blood pressure in the 10-week-old stroke-prone spontaneously hypertensive rats on a high sodium diet could be delayed significantly by sinorphan treatment. It was previously demonstrated that chronic administration of the NEP inhibitor SCH 34826 prevents hypertension in NaCl-sensitive spontaneously hypertensive rats (SHR-S) by increasing endogenous ANP (31). These NaCl-sensitive SHR-S received SCH 34826 for 4 weeks beginning immediately before the initiation of an 8% NaCl diet. SCH 34826 had no effect on blood pressure in SHR-S fed a normal diet (31).

These results were also underlined by studies providing evidence that chronic administration of the NEP inhibitors SCH 34826 and SCH 42495 in adult SHR with established hypertension for 1 month produced no significant changes in blood pressure

(32, 33). These findings agree with our results that chronic oral administration of a NEP inhibitor prevents NaCl-sensitive hypertension in SHR and support the hypothesis that NaCl-sensitive hypertension in SHR-S and SHR-SP is an ANP-deficient state.

Long-term treatment with sinorphan in SHR-SP on a high-sodium diet not only prevented the development of hypertension; it also prevented the associated cardiac and renal hypertrophy. There is some evidence that the reductions in cardiac and kidney mass are independent of the hemodynamic changes in these rats. It was previously reported that chronic treatment for 1 month with the NEP inhibitors SCH 34826 and SCH 42495 in adult SHR with manifest hypertension reduced cardiac mass and the amount of fibrotic tissue present in the left ventricle despite the lack of antihypertensive activity (32, 33).

It has been proposed that ANP acts as a physiological antagonist of the renin-angiotensin-system (1–3, 28). The left ventricular myocytes and the cells within the kidney contain detectable amounts of angiotensin II that may act as a trophic growth-promoting factor. Thus, local inhibition of angiotensin II by enhanced ANP activity in ventricles and kidneys may exert a protective and anti-hypertrophic influence on cardiac myocytes and kidney cells. This interpretation, relying on a possible interaction between ANP and angiotensin II, does not rule out other mechanisms, still unexplored, that may have played a part in determining changes in cardiac hypertrophy. NEP hydrolyzes numerous substances including brain natriuretic peptide, bradykinin, and substance *p* (34). The role of these peptides in ventricular and kidney hypertrophy remains to be elucidated.

At the end of treatment, in the plasma compartment no significant decrease in renin activity, angiotensin I and aldosterone concentration was observed in the sinorphan group despite elevated ANP plasma levels. A slight protective effect on kidney function is reflected in the reduced urea concentration in plasma in the sinorphan group. Treatment with sinorphan led to a significant improvement in the overall health of SHR-SP on a high sodium diet as demonstrated by a continuous gain in body weight and an increased survival rate.

These results of the second study demonstrate a substantial improvement of cardiac pump function and ventricular hypertrophy in old SHR-SP with comprised cardiac function by two-week inhibition of the neutral endopeptidase 24.11 with sinorphan.

At the time treatment commenced, the SHR-SP were in a stage of disease, characterized by declining arterial pressure, marked increases in end-diastolic filling pressure, cardiac hypertrophy, and high plasma ANP. Compared with normotensive Wistar Kyoto rats of similar age, plasma ANP in the old SHR-SP was elevated five-fold (35). Also, basal plasma renin activity was high. Similar pathological signs have been observed in rats with congestive heart failure after myocardial infarction (36).

The marked reduction in end-diastolic pressure obviously lowered cardiac load, because heart weights were substantially reduced in spite of the short duration of treatment. Sinorphan might have lowered end-diastolic pressure through both a reduction in pre- and afterload and circulating volume, because ANP increases venous distensibility (37), can reduce total peripheral resistance (38), and, at least acutely, produces volume contraction (39). This occurred although systolic blood pressure was not reduced.

We found no sustained natriuretic effect after sinorphan. However, neither chronic enkephalinase inhibition nor ANP have been shown to be persistently natriuretic or diuretic (40-42). This is presumably so, because chronic ANP is natriuretic only in chronically hypervolemic states (43, 44). Other characteristic signs of ANP action, such as an increase in plasma and urinary cGMP as well as a reduction in plasma renin activity were noted in this experiment (1-3, 28, 45).

In conclusion, the results of the present studies demonstrate that long-term NEP inhibition with sinorphan has inhibitory effects on malignant hypertension and associated cardiac hypertrophy in young SHR-SP on a high-sodium diet and produces substantial improvements in cardiac pump function and ventricular hypertrophy in old SHR-SP with compromised cardiac function.

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