Restorative Effect of Atrial Natriuretic Peptide or Chronic Neutral Endopeptidase Inhibition on Blunted Cardiopulmonary Vagal Reflexes in Aged Rats

Colleen J. Thomas, Robin M. McAllen, Lauren M. Salo, Robyn L. Woods

Abstract—Arterial baroreflex function diminishes with age, but whether cardiopulmonary vagal reflexes are similarly altered with physiological aging has not been fully elucidated. In this study, predominantly cardiac high pressure mechanoreceptor-activated (ramp baroreflex) and cardiopulmonary chemoreceptor-activated (von Bezold-Jarisch reflex) vagal reflexes in conscious, instrumented rats were impaired by 30% to 40% (P<0.05) in 24-month-old rats compared with 6-month-old rats (n=12). To determine whether this is a restorable deficit, the influence of atrial natriuretic peptide (ANP), either by infusion or blockade of its breakdown, was studied. ANP infusion was previously shown to enhance Bezold-Jarisch reflex and ramp baroreflex bradycardia in young adult rats. The present study confirmed that vagal reflex augmentation by ANP (50 pmol/kg per minute) also occurs in old rats (increased by 60±18% [Bezold-Jarisch reflex] and 91±15% [ramp baroreflex; P<0.05]). Direct vagal stimulation in anesthetized animals showed that the target for ANP was not the cardiac vagus itself in old rats (n=7), although in young rats only, we confirmed the published finding that ANP enhances vagal bradycardia (by 58±14%, n=7). Neutral endopeptidase 24.11 degrades ANP and several other peptides. The neutral endopeptidase inhibitor candoxatrilat (5 mg/kg per day IV for 7 to 9 days) restored vagal reflex bradycardia in old rats (n=6) to levels similar to those in young neutral endopeptidase inhibitor-treated rats (n=6). Impaired cardiopulmonary vagal reflex control of heart rate is thus a feature of normal aging, and this deficit may be ameliorated by either ANP infusion or chronic neutral endopeptidase inhibition. (Hypertension. 2008;52:696-701.)

Key Words: aged ■ autonomic ■ cardiac ■ ischemia ■ sensory afferent

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iminished arterial baroreflex control of heart rate (HR) is a well-documented feature of aging (see reviews1-2), but surprisingly few studies have examined the effect of aging on reflexes originating in the heart. It is generally accepted that low-pressure cardiopulmonary reflexes are less effective with advancing age and that this may play a role in the decreased ability of the elderly to regulate blood volume and maintain blood pressure during gravity challenges.3,4 Whether other cardiocirculatory reflexes such as the cardiopulmonary chemoreflex (von Bezold-Jarisch [BJR]) or those activated by high-pressure ventricular stretch receptors (ramp baroreflex) are also compromised with age is not clear. The BJR is thought to be cardioprotective (promoting reflex bradycardia, hypotension, and vasodilatation) and is activated by 5-HT1 receptors on ventricular vagal afferents.5 Davidow and colleagues concluded that the BJR in conscious 14- and 24-month-old rats was reduced compared with 6-month-old rats,6 whereas Sakima and colleagues7 reported that the BJR was not diminished in older (16- to 20-month-old) rats.

The potential for cardioprotection by enhancing parasympathetic activity has long been recognized and this avenue is underexploited by current therapies.8 In young animals, acute administration of atrial natriuretic peptide (ANP) enhances not only the BJR,9,10 but also the reflex bradycardia after cardiopulmonary high-pressure baroreceptor activation by the rapid “ramp” method,9,11,12 an effect still evident in sinoaortic denervated rats.12 The influence of ANP on cardiopulmonary vagal afferent reflexes in aging has not been previously reported. As a peptide, ANP has limited clinical potential due to its rapid gastrointestinal inactivation when administered orally, whereas inhibiting the enzyme responsible for the metabolism of natriuretic peptides (neutral endopeptidase [NEP], EC 24.11) potentiates renal and hemodynamic effects of the peptide clinically and experimentally.13,14

The present study therefore determined if acute increases in synthetic ANP, and chronic increases in endogenous natriuretic peptides after NEP inhibition, enhance cardiopulmonary vagal reflex function in young and old rats. The study aims were: (1) to assess whether cardiopulmonary vagal reflexes are diminished with age in conscious rats; (2) to test whether the effect of acute infusions of ANP on these cardiopulmonary vagal reflexes is preserved in aged rats; (3)
to determine whether vagal efferent neurotransmission is a target for this action of ANP; and (4) to examine whether chronic administration of a NEP inhibitor (candoxatrilat) influences vagal reflexes in old or young rats.

Methods

General
This study was approved by the Howard Florey Institute Animal Ethics Committee and performed in accordance with the Australian Guidelines for Care and Use of Laboratory Animals. Cardiac vagal reflexes were examined in male Munich-Wistar (Animals Resources, Perth, Australia) conscious rats, chronically instrumented with arterial and venous catheters, and allocated to 1 of 4 groups: 6-month-old rats (n=12, age range 6 to 7 months), 24-month-old rats (n=12, age range 23 to 26 months), NEP inhibitor-treated 6-month-old rats (n=6, age range 6 to 7 months), and NEP inhibitor-treated 24-month-old rats (n=6, age range 23 to 25 months). Nonrecovery, vagal stimulation experiments were carried out in further anesthetized 6-month-old (n=7) and 24-month-old (n=7) Munich-Wistar rats.

Surgical Preparation for Chronic Instrumentation
Surgical procedures were the same as those reported previously,9 except that the general anesthesia used for instrumentation was isoflurane (2%). Briefly, 2 vascular cannulae were implanted in each animal with the free ends of the cannulae tunneled subcutaneously and exteriorized behind the neck. The cannula in the abdominal aorta was used for measuring arterial blood pressure and HR. The other cannula in the right jugular vein (and positioned near the right atrium) contained a triple lumen, which allowed simultaneous infusions of vasoactive drugs or ANP separately into each lumen. This cannula was also used for NEP inhibitor treatment.

NEP Inhibitor Treatment in Conscious Rats
Candoxatrilat (UK 73,967; Pfizer, Sandwich Laboratories, Kent, UK), a specific inhibitor of NEP, was administered daily (5 mg/kg per day intravenously in 0.5 mL saline over a 1-hour period) for 7 to 8 days beginning 1 day after instrumentation surgery and ending on the day of reflex testing, immediately before the start of the experiment. Candoxatrilat is the active product of the orally active Candoxatril, which has been used in numerous studies in humans15,16; a lower dose of Candoxatrilat (3 mg/kg) was reported to inhibit renal NEP binding in rats for up to 24 hours.17

Experimental Protocol for Reflex Measurements
Experiments were performed in conscious, unrestrained rats 1 week after instrumentation with an aged rat always paired with a younger one on a given day. At the start of each experiment, the arterial cannula was connected to a transducer (Cobe, Lakewood, Colo) and HR was measured using a tachograph (Baker Institute, Melbourne, Australia) triggered from the phasic blood pressure signal. Arterial pressure and HR were continually recorded at a sampling rate of 200 Hz using the AcqKnowledge data acquisition system (Biopac Systems, Goleta, Calif), which calculated mean arterial pressure (MAP). Cardiopulmonary vagal reflexes were tested in the presence of ANP (50 pmol/kg per minute intravenously infusion; Bachem AG, Bubendorf, Switzerland) or saline vehicle (390 μL/h) in alternate order. See the online data supplement at http://hyper.ahajournals.org for additional details.

HR Reflex Techniques
HR reflex testing methods in rats have been described previously in detail.11,12,14,19 Briefly, (1) ramp baroreflex responses were assessed after rapid MAP increases of approximately 50 mm Hg evoked by methoxamine (100 μg/kg intravenously; Sigma Chemical) aiming for a similar rate of MAP rise across all animals (approximately 20 mm Hg/s). Linear regression analysis was applied to the progressive HR responses to MAP changes. For full details, see Figure S1). Three replicate ramp tests were performed in each rat in the absence and presence of ANP, allowing full baseline recovery between tests; (2) BJR bradycardia and hypotension were measured to 3 bolus doses of serotonin (5-HT; 2 to 18 μg/kg intravenously; Sigma Chemical). The 3 5-HT doses were individually chosen for each rat to give threshold, intermediate, and submaximal responses; once chosen, they were maintained in that animal for tests both in the absence and presence of ANP. At least 10 minutes were allowed between successive 5-HT doses. For further details, see Figure S2.

ANP Measurements
Blood samples were collected from conscious rats after HR reflex testing had been completed and while ANP or saline vehicle was still being administered. The samples were centrifuged and the plasma was stored at −20°C until the time of analysis by radioimmunoassay (for details, see the data supplement). At the end of experiments in 6 young and 5 old rats, hearts were collected immediately after barbiturate overdose (Euthatal; pentobarbitone sodium, 350 mg/mL IV; Rhone Merieux). Atria and ventricles were separated, weighed, fresh-frozen in liquid nitrogen, and stored at −20°C for subsequent measurement of ANP content using the radioimmunoassay described for plasma.

Vagal Stimulation Experiments
Under general anesthesia (2% isoflurane), both cervical vagi were cut and the right vagus was prepared for stimulation. Propranolol (1 mg/kg, repeated as required; Sigma Chemical) was given to block sympathetic actions on the heart. The caudal end of the right vagus was stimulated with 10-second supramaximal trains at a range of frequencies, and the maximum increase in cardiac interval was measured in each case. Stimulus frequency–response relationships were measured before and at least 20 minutes after the onset of ANP infusion (50 pmol/kg per minute IV). At the completion of the experiment, the rat was euthanized with an overdose of barbiturate. For full details, see the online data supplement.

Data Analysis
The effects of age on cardiopulmonary reflex bradycardia and hypotension were determined from 2-way analysis of variance with repeated measures across doses of 5-HT (BJR) or replicate baroreflex ramps in untreated (n=12 in each group) or in NEP inhibitor-treated rats (n=6 in each group). The effect of ANP on vagal reflexes was determined by comparing the within-animal bradycardic responses to the same doses of 5-HT (BJR) or triplicate ramps (baroreflex) without and during ANP infusion by 2-way analysis of variance with repeated measures with a Bonferroni adjustment of the α for multiple comparisons. To determine whether the ANP effect was different between age groups, the additional effect of ANP (ie, the difference between reflex bradycardia during baseline [vehicle infusion] and during ANP infusion) in young and old rats was compared by 2-way analysis of variance.

The effects of age and ANP infusion on the efferent cardiac vagal activity were determined from the linear regression analysis of the relationship between stimulation frequency (up to 20 Hz) and the bradycardic response at each frequency step. Slopes were compared with 2-way analysis of variance. Baseline hemodynamics or ANP levels were analyzed for the effects of age or NEP inhibitor treatment by one-way analysis of variance. For all tests, P<0.05 was taken as the level of significance. Data are mean±SEM unless otherwise stated.

Results

Body Weight and Resting Hemodynamics in Young and Old Rats
Twenty-four-month-old Munich-Wistar rats were approximately 12% heavier than 6-month-old rats (461±12 g versus 411±5 g, respectively, P<0.05). NEP inhibitor treatment did
not affect body weight in either old (467±19 g) or young (419±10 g) rats. There was no significant difference in resting MAP or HR between old and young rats (113±2 versus 114±2 mm Hg and 310±3 versus 309±4 beats/min, respectively; n=12 in each group).

Cardiopulmonary Vagal Reflexes Were Attenuated in Old Rats

von Bezold-Jarisch Reflex
In 6-month-old rats, the 3 doses of 5-HT that produced threshold, intermediate, and submaximal reflex bradycardia were 4.6±0.3, 7.9±0.7, and 11.2±0.9 µg/kg with an average dose of 8.0±0.6 µg/kg. Similar doses of 5-HT were administered to 24-month-old rats with an average dose of 5-HT of 7.7±0.7 µg/kg. Bradycardic responses across the 3 doses of 5-HT were significantly (P<0.05) attenuated in old compared with young rats (Figure 1A). The mean reflex change in heart period across all doses was 228±24 ms in young rats and 155±23 ms in old rats, a 32% decline with age. There was no significant difference in hypotension to 5-HT doses between age groups (−11±5 mm Hg in old versus −14±5 mm Hg in young rats).

Ramp Baroreflex
Methoxamine-induced “ramp” blood pressure rises in each age group were similar (19±1 mm Hg/s in old versus 22±1 mm Hg/s in young rats), and regression analyses relating change in HR to change in MAP all showed r² values >0.93. Mean ramp gain in 6-month-old rats was −2.12±0.21 beats/min per mm Hg (Figure 2A), similar to previous measurements in 3-month-old animals,12,18 whereas in 24-month-old rats, it was −1.30±0.22 beats/min per mm Hg (Figure 2B, P<0.05), representing a 39% decline with age.

Is the Effect of ANP Infusion Preserved in Old Rats?

Baseline Hemodynamics
Acute ANP infusion reduced resting MAP in both old (−9±4 mm Hg) and young (−7±2 mm Hg) rats (n=12, P<0.05 for both groups). Baseline HR was unaffected by ANP infusion in either group.

von Bezold-Jarisch Reflex
In young rats, ANP significantly enhanced the reflex increase in heart period from 228±24 to 330±37 ms (within-animal comparison over all doses, P<0.05). In old rats, ANP also enhanced the reflex increase in heart period from 155±24 to 248±31 ms (P<0.05). The reflex changes to 5-HT doses before and after ANP are compared in Figure 3. The accompanying reflex hypotension was not different in the presence of ANP (−12±5 mm Hg in old and −18±8 mm Hg in young rats) compared with saline infusion (see previously) in either age group. For further details, see Figure S3A.

Ramp Baroreflex
In both age groups, the methoxamine-induced rates of rise in MAP during ANP infusion were similar to control conditions. In 6-month-old rats, ANP infusion significantly (P<0.05) increased ramp baroreflex gain to −3.20±0.24 beats/min
inhibitor-treated rats (8.2 nmol/g wet weight in old rats, n=6) and by 130% (approximately 3-fold compared with untreated rats) was found in both age groups. ANP content was 198 nmol/g wet weight in old rats (n=6) and 21 nmol/g wet weight in young rats (n=6). Right atrial tissue ANP content was 391 nmol/g wet weight in both age groups. The combined ventricular tissue ANP concentration was 803 ± 296 nmol/g wet weight in young rats and 411 ± 64 nmol/g wet weight in old rats.

**Cardiac Vagal Neurotransmission**

Unlike in the conscious state (see previously), resting HR (277 ± 10 beats/min) of old rats under anesthesia was significantly (P<0.05) lower than in the younger rats (320 ± 9 beats/min). This difference was maintained after vagotomy and propranolol, showing that old rats had a lower intrinsic heart rate than young rats (241 ± 7 versus 282 ± 14 beats/min; P<0.05) as is the case for older humans.20 The bradycardia response to efferent vagal stimulation at up to 20 Hz closely fitted a linear relationship with r² values > 0.96 in all animals. This relation showed no significant difference in slope between old and young rats (β = 2.47 ± 0.31 beats/min per mm Hg; Figure 2D, a 91% increase). For further details, see Figure S3B.

**Effect of ANP on Cardiac Vagal Neurotransmission in Old Rats**

ANP infusion significantly (P<0.05) enhanced the slope of the relationship in young rats (β = 4.74 ± 0.49 beats/min per Hz; Figure 4, left graph) but not in old rats (Figure 4, right graph).

**Effect of ANP on Heart Rate**

ANP infusion significantly (P<0.05) enhanced the slope of the relationship in young rats (β = 4.74 ± 0.49 beats/min per Hz; Figure 4, left graph) but not in old rats (Figure 4, right graph). ANP infusion in old rats (β = 2.47 ± 0.31 beats/min per mm Hg) did not differ significantly from that in young rats (β = 4.74 ± 0.49 beats/min per mm Hg). This difference was maintained after vagotomy and propranolol, showing that old rats had a lower intrinsic heart rate than young rats (241 ± 7 versus 282 ± 14 beats/min; P<0.05) as is the case for older humans.20 The bradycardia response to efferent vagal stimulation at up to 20 Hz closely fitted a linear relationship with r² values > 0.96 in all animals. This relation showed no significant difference in slope between old and young rats (β = 2.47 ± 0.31 beats/min per mm Hg; Figure 2D, a 91% increase). For further details, see Figure S3B.

**Effect of Age on Plasma and Heart ANP Levels**

**Plasma**

Resting plasma ANP levels tended to be higher (P=0.06) in 24-month-old rats (80 ± 11 fmol/mL, n=7) compared with 6-month-old rats (46 ± 10 fmol/mL, n=10). Acute ANP infusion increased these levels by 85 ± 34 fmol/mL in old rats and by 130 ± 52 fmol/mL in young rats. Chronic NEP inhibitor treatment did not significantly change resting plasma ANP levels in either age group (65 ± 14 fmol/mL in old rats, n=5; 67 ± 5 fmol/mL in young rats, n=6). Infusions of ANP into NEP inhibitor-treated animals raised plasma ANP levels by 273 ± 68 fmol/mL in old rats and by 327 ± 144 fmol/mL in young rats. This greater increase in plasma ANP (approximately 3-fold compared with untreated rats) was significant (P<0.05, n=11).

**Heart**

ANP levels in heart tissues were not different between 24-month-old and 6-month-old rats. Left atrial tissue ANP content was 198 ± 68 nmol/g wet weight in old rats (n=5) and 231 ± 21 nmol/g wet weight in young rats (n=6). Right atrial tissue ANP content was 391 ± 96 nmol/g wet weight in old rats and 411 ± 64 nmol/g wet weight in young rats. Combined ventricular tissue ANP concentration was 803 ± 296 nmol/g wet weight.
pmol/g wet weight in old and 775 ± 217 pmol/g wet weight in young rats.

**Discussion**

The present study demonstrates that old rats (24-month-old) have a deficit in the ramp baroreflex bradycardia (approximately 39%) and a quantitatively similar deficit in the BJR (approximately 32%) compared with younger but mature adults (6-month-old). For the first time, this study provides clear evidence that manipulation of the natriuretic peptide system, either acutely with infusion of ANP or chronically (although less specifically) with NEP inhibition, improves cardiopulmonary vagal reflex function in old rats.

Munich-Wistar rats were chosen for these studies because they do not become obese with age with ad libitum feeding, unlike many other rat strains.\(^{21,22}\) Although not possessing longitudinal data on cardiopulmonary reflex changes, our present and previous findings in Munich-Wistar rats suggest that age-associated deficits in HR reflexes progress through present and previous findings in Munich-Wistar rats suggest that age-associated deficits in HR reflexes progress throughout the lifetime of the animal. Cardiac baroreflex sensitivity falls between 3 and 6 months of age (previous study) and diminishes further still between 6 and 24 months of age. For the present study, 6-month-old rats were chosen as the younger control because at this age, they have reached full maturity, thereby minimizing the potentially confounding influences of body size and growth that occur during adolescence. The use of chronic instrumentation allowed all reflex studies to be done in awake, quiet, and unstressed animals, thereby ensuring that the cardiovascular reflex measurements were carried out under optimal conditions.

What is the cause of reduced cardiopulmonary reflex function with age? Neural deficits may contribute to diminished autonomic reflex function with age. Efferent autonomic dysfunction is generally not favored as an explanation for the loss of vagal tone and responsiveness.\(^{23}\) End-organ autonomic receptors appear to be intact, and our present results confirmed that age caused no diminution in cardiac responsiveness to vagal stimulation\(^{24}\) (Figure 4). Age-related loss of cardiac reflex function may therefore result from changes in (1) the sensory receptors; (2) the afferent nerves; or (3) central nervous processing (as occurs for arterial baroreflexes).\(^{25}\) Our present data cannot distinguish which nor can they tell us whether natriuretic peptides are involved (see subsequently).

Regardless of the underlying mechanisms of the age-related deficit, the ameliorating effect of systemic ANP would be due to an action outside the blood–brain barrier at the level of the vagal afferents\(^{8–12}\) or possibly the sensory circumventricular organs.\(^{26}\) In young rats only, we confirmed Atchison and Ackermann’s observation\(^{27}\) that ANP may enhance cardiac responsiveness to vagal efferent stimulation. This effect was not present in old rats, yet they showed reflex enhancement by ANP comparable with that of young rats. In old rats at least, the major site of ANP’s action cannot be efferent. Interestingly, we recently found that under similar conditions, BNP infusion in young adult Wistar rats enhanced the BJR, yet had no effect on vagal efferent responsiveness.\(^{28}\)

Because additional ANP restored the vagal reflex deficit in old rats, we considered the hypothesis that normal circulating levels of ANP may decline with age. However, as other studies have shown in different rat strains,\(^{21,29}\) and indeed in humans,\(^{30,31}\) plasma ANP levels in old Munich-Wistar rats were increased approximately 2-fold compared with younger rats. These elevated plasma levels are most likely due to decreased clearance\(^{31}\) because cardiac levels of ANP were not enhanced in old rats.

So why do the elevated circulating ANP levels not sustain normal reflex bradycardic activity in old rats? To our knowledge, alterations in natriuretic peptide receptor expression have not been examined in aging. One possibility is that some reduction in natriuretic peptide receptor number or action prevents the endogenous ANP (and other natriuretic peptides) from maintaining cardiac reflex function in old rats. Against this argument is the observation that exogenous ANP, at doses that raise plasma levels approximately 10-fold, are as effective in old as in young rats. This does not, however, rule out the possibility that old rats have some modest deficit in the basal level of natriuretic peptide signaling, which we have shown to be necessary for the full expression of the BJR.\(^{19}\) Many other mechanisms could underlie the reduced reflex responsiveness of older rats, however, and the issue awaits direct study.

The finding that endogenous ANP levels did not rise after 1 week of Candoxatrilat treatment is not uncommon. Other studies have shown that with chronic NEP inhibitor treatment, ANP levels re-establish at a steady-state level where release falls, compensating for the reduced clearance.\(^{32,33}\) NEP inhibitor treatment markedly reduced the breakdown of exogenous ANP as demonstrated by potentiated plasma ANP levels measured in both age groups of rats when the peptide was infused.

Although candoxatrilat is a specific inhibitor of NEP, the enzyme acts on a number of substrates in addition to ANP and other natriuretic peptides. Our focus was on ANP in the present study, but BNP, and to a lesser extent CNP, have similar actions on cardiopulmonary vagal reflex function\(^{10,11}\) so the effects of NEP inhibition may involve all natriuretic peptides. Chronic NEP inhibition may also act through other NEP substrates, which include adrenomedullin, bradykinin, substance P, and endothelin.\(^{34–36}\) There is little evidence that adrenomedullin or endothelin influences cardiopulmonary reflex function, but bradykinin and substance P have been shown to enhance the BJR.\(^{37,38}\) Thus, NEP inhibition could enhance cardiopulmonary vagal reflexes in old rats also by non-natriuretic peptide actions.

In summary, cardiac vagal afferent-driven baro- and chemoreflex functions were reduced in elderly Munich-Wistar rats. These reflexes remained sensitive to the enhancing action of ANP, however, and chronic NEP inhibition was able to abolish the deficit in old rats compared with young adults. Enhanced availability of endogenous natriuretic, and perhaps other peptides, presumably underlies the restorative effect of NEP inhibition.

**Perspectives**

The vagal reflexes studied here may be considered to be cardioprotective and would act to unload the heart during periods of ischemia or excessive arterial pressure. Natriuretic
peptides enhance those actions, an effect that survives into old age when it may be most needed. Furthermore, natriuretic peptide enhancement of reflexly activated parasympathetic activity may shift the sympathovagal balance to prevent or delay adverse cardiovascular events and mortality caused by increased sympathetic drive in apparently healthy elderly humans.

Acknowledgments
We thank Tony Dornom for his expert technical assistance with surgical preparation of the animals and Greg Thomas and Tom Vale for the long-term care and maintenance of the animals. Candoxatril was a generous gift from Pfizer Global Pharmaceuticals, Australia.

Source of Funding
This study was supported by a block grant from the National Health and Medical Research Council of Australia (No. 983001).

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Hypertension. 2008;52:696-701; originally published online August 25, 2008;
doi: 10.1161/HYPERTENSIONAHA.108.111302

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

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/52/4/696

An erratum has been published regarding this article. Please see the attached page for:
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The correct title has been used in the final print version of the article in the October 2008 issue of the journal (Hypertension. 2008;52:696–701) and in the current online version.
THE RESTORATIVE EFFECT OF ANP OR CHRONIC NEP INHIBITION ON BLUNTED CARDIOPULMONARY VAGAL REFLEXES IN AGED RATS

Colleen J. Thomas, Robin M. McAllen, Lauren M. Salo and Robyn L. Woods

Howard Florey Institute,
The University of Melbourne,
Victoria, Australia, 3010.
METHODS

Experimental Protocol

On a given experimental day, HR reflexes were assessed in one aged rat matched with one young rat, whenever possible. Cardiopulmonary reflexes were tested in the following order: 1) ramp+ANP, 2) BJ reflex + ANP, 3) BJ reflex + saline and 4) ramp + saline, with the order of ANP or saline infusion alternated between animals from each age group. Saline vehicle or ANP (50 pmol/kg/min) was infused for 20 mins to reach steady-state levels in the circulation before HR reflex testing commenced.

HR reflex techniques

We have previously shown that ANP selectively enhances ramp baroreflex bradycardia but not ‘steady-state’ arterial baroreflex activity\(^1\textsuperscript{-3}\). In the present study, we have used a method of rapid ramp rise in arterial blood pressure, which is a modification of the beat-to-beat analysis method of Struyker-Boudier and colleagues\(^4\), to activate cardiac and arterial mechanoreceptor reflexes. Rapid infusions of phenylephrine (‘ramp’) evoke marked bradycardic responses in sino-aortically denervated animals\(^5,6\), a reflex response that cannot be attributed to arterial baroreceptor activation in those animals. Briefly, the rats were given fast (over 4 to 6 s) infusions of the peripherally acting vasoconstrictor agent methoxamine (~100 µg/kg doses; Sigma, St Louis, Missouri, USA), resulting in rapid increases in BP and falls in HR (see Figure S1A). Absolute increases in BP using the rapid ‘ramp’ method were in the order of 50-60 mmHg. To determine reflex responsiveness, the relationship between HR and BP data, collected on a beat-to-beat basis over a defined range, was determined. For consistency of analysis between ramp responses, and indeed between animals, an initial rise in BP of 5 mmHg from baseline readings was used as the marker, from which a +45 mmHg change in BP was then used to analyse the reflex responses. To obtain the best correlation
between HR responses to BP changes, linear regressions of HR versus MAP at 0.5s earlier than the corresponding HR value were used, which is consistent with the delay used by others and ourselves previously with this analysis technique in rats. HR and BP data were well fitted to linear regression analysis, with \( r^2 > 0.93 \) in all cases, and with the slope giving an indication of the gain or baroreflex sensitivity. In each animal, 3 replicate ramp tests were performed and used as repeated measures for analysis.

Cardiopulmonary chemosensitive afferents were activated by bolus injections of 5-HT (serotonin, creatinine sulphate complex, Sigma Chemical Co.) in the range 2-18µg/kg i.v. Stimulation of these receptors (by the so-called von Bezold Jarisch reflex or BJR) resulted in rapid (within 5 to 10s) dose-dependent falls in HR, associated with a fall in BP (see Figure S2). Changes in BP to 5-HT were taken at the same time as the maximum bradycardic responses. BJR bradycardia generally begins within 5s after 5HT injection, reaches a maximum within 1-2s, and is sustained for a further 2-3 s, occasionally longer at the highest doses (see examples in Figure S2). Changes in HR or BP outside the 10s window after 5-HT was administered were not included in the measurements due to possible non-reflex (vascular) effects of 5-HT. Three doses of 5-HT, selected from a minimum of 5 doses that were administered, were used to cover the range of responsiveness from just above threshold (‘low’ dose), to intermediate (‘medium’ dose), to near maximal (‘high’ dose) in each animal. The exact doses of 5HT varied between rats because of different sensitivities. In individual rats, however, identical doses of 5-HT were given in the presence and absence of ANP.

**Vagal stimulation experiments**

Under 2% isofluorane anesthesia, the rat was tracheostomized, ventilated with positive pressure at a rate of 60/min and at a tidal volume sufficient to suppress spontaneous respiration. The right jugular vein was cannulated for administration of drugs including ANP.
and infusion of maintenance fluids (Haemaccel, 1ml/h). A femoral artery was cannulated for arterial blood pressure recording. Silver electrode wires were implanted under the skin for recording of the Einthoven’s 3-lead EKG. The animals were heated by an electric blanket to maintain rectal temperature between 36.5 and 37.0°C. Both vagi were dissected free in the neck and cut cranially. The caudal segment of the right vagus was placed over a pair of silver wire hook electrodes under a pool of mineral oil. The level of voltage used to supramaximally stimulate the right vagus was determined using 10 Hz stimulus trains at the beginning of the experiment (usually 5-10v). To minimize confounding influences from reflex changes in sympathetic drive, propranolol was given before measurements were made. This lowered resting HR by 78±7 bpm in young rats and by 56±7 bpm in old rats (~20%). The vagus was stimulated in 10s trains at 1,2,5,10,20,40,80 Hz, curtailed at the higher stimulus frequencies if they caused asystole. At the highest stimulation frequencies atrioventricular block was common: this potential confound was avoided by discarding all data from 40 and 80 Hz stimulations. Sufficient time was always allowed between trains for HR to recover fully to its steady-state level.

**ANP measurements**

To determine the effect of age and NEP inhibitor treatment on plasma ANP levels, 2 ml arterial blood samples were collected into pre-chilled EDTA tubes and centrifuged for 10 mins at 2500 rpm and 4°C. The effectiveness of NEP inhibition to reduce plasma clearance of ANP was tested by comparing plasma ANP levels reached during steady-state infusion of ANP in NEP-inhibitor-treated rats and in vehicle-treated rats. For each rat, the volume of blood removed for sampling was replaced with an equal volume of the plasma expander Haemaccel (Behring, Marburg, Germany). Plasma ANP concentrations were determined following duplicate sample extraction procedures previously described 8. Briefly, ANP was extracted from 0.5ml aliquots of plasma, and eluted from Sep-Pak C18 cartridges (Waters,
Milford, MA) with 70% methanol + 0.1% TFA. Samples were dried under air and stored at -80°C until assay. Each dried plasma sample was reconstituted in 0.5ml assay buffer for assay. ANP was also extracted from myocardial tissue according to the method of Younes and colleagues. Briefly, for each animal, atrial and ventricular tissue was boiled in 10 vol acetic acid (0.1M), Triton X-100 (0.1%), PMSF (1µg/ml) and pepstatin (1µg/ml). After cooling, the tissue was homogenized and centrifuged at 22,000g for 30 min (4°C). 100µl aliquots of supernatant were assayed by radioimmunoassay as described below.

Radioimmunoassay

The radioimmunoassay for ANP was performed using commercial rat ANP antiserum (Peninsula Laboratories, San Carlos, CA), 125I-rat ANP tracer (Peninsula Laboratories, San Carlos, CA) and the same rat ANP standard used in the experiments (Bachem AG, Bubendorf, Switzerland). Recovery of 125I rat ANP added to plasma prior to extraction was approximately 75-85%. The limit of detection was <2 fmol/ml. Inter-assay coefficient of variation (CV) was 10% and the intra-assay CV was 5%. All samples from each animal were assayed in the same radioimmunoassay in duplicate to reduce between-measurement variation.

RESULTS

Figure S1A illustrates the individual ramp baroreflex responses in young (left panel) and old (right panel) rats. Figure S1B shows the corresponding plots and regression lines relating heart rate to mean arterial pressure. Note the tightness of fit.

Figure S2 shows representative examples of BJR responses to low, medium and high doses of 5HT in a young and an old rat.

Figure S3A shows grouped data of BJR responses in young and old rats: in both age groups ANP significantly (P<0.05) enhanced the BJR over the 3 doses of 5-HT. Figure S3B
shows grouped data from the triplicate ramp tests in young (left) and old (right) rats. ANP significantly (P<0.05) enhanced ramp gain in both age groups.

REFERENCES


Figure S1. ‘Ramp’ baroreflex in conscious young and old rats. (A) Example of cardiopulmonary baroreflex-induced responses in pulsatile arterial blood pressure and heart rate to rapid (ramp) injections of methoxamine (Mx; 100 µg/kg) into the jugular vein in individual 6-month old (‘young’) and 24-month old (‘old’) Munich-Wistar rats. (B) Plot of corresponding data and regression lines relating heart rate to ramp changes in blood pressure from those same individual rats.
Figure S2. Representative von Bezold-Jarisch reflex responses in conscious young and old rats. Dose-related heart rate and pulsatile arterial blood pressure responses measured with 3 doses of serotonin (5-HT) into the jugular vein in 6-month-old (‘young’) and 24-month-old (‘old’) Munich-Wistar rats.
Figure S3. Effect of ANP infusion on Bezold-Jarisch reflex (BJR) and ramp baroreflex responses in conscious young and old rats. A, Heart period responses to the BJR tested over 3 doses of 5-HT (‘low’ ~4 μg/kg, ‘med’, medium ~8 μg/kg and ‘high’ ~12 μg/kg i.v.) in the absence (open symbols) and presence of ANP infusion (gray symbols, 50 pmol/kg/min) in 6-month-old Munich-Wistar rats (left panel, n=12) and 24-month-old Munich-Wistar rats (right panel, n=12). B, Ramp baroreflex sensitivity to 3 replicate doses of methoxamine (“1, 2, 3”, each dose 100μg/kg, IV) per animal in the absence (unhatched bars) and presence of ANP infusion (hatched bars) in 6-month olds rats (no shading, n=12) and 24-month old rats (gray shading, n=12). * Significant effect of ANP compared to saline infusion from 2-way ANOVA with repeated measures and Bonferroni adjustment of α for multiple comparisons (P<0.05).