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ORIGINAL ARTICLE

Enantioselective pharmacokinetics and cardiovascular effects of nebivolol in L-NAME hypertensive rats

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The cardiovascular effects and pharmacokinetics of nebivolol were assessed in N(G)-nitro-l-arginine methyl ester (L-NAME) hypertensive and normotensive control rats. Male Wistar rats were randomly divided to drink tap water (control) or L-NAME solution for 2 weeks. The effects of nebivolol (3 or 10 mg kg^{-1} i.v.) on blood pressure (BP), heart rate and BP variability (BPV) were recorded in awake L-NAME and control rats. Short-term and beat-to-beat BPV was assessed by the s.d. and spectral analysis of the BP recordings. Nebivolol pharmacokinetics was studied by means of traditional blood sampling. Nebivolol showed enantioselective pharmacokinetics in both experimental groups; the clearance and the volume of distribution of *l*-nebivolol were significantly greater than those of the *d*-enantiomer. The hypotensive response to nebivolol was significantly enhanced in L-NAME rats (Δmean arterial pressure (MAP): $-16.1 \pm 1.1\%$, $P<0.05$ *vs.* control rats) compared with normotensive animals (ΔMAP: $-1.4 \pm 2.1\%$). An analysis of the beat-to-beat BPV showed a greater reduction in VLF BPV in the L-NAME compare with the control rats. Nebivolol significantly reduced the low-frequency/high-frequency ratio in hypertensive L-NAME animals compared with normotensive rats. Short-term BPV was markedly reduced by nebivolol in both experimental groups, although the attenuation of the s.d. of BP recording was greater in L-NAME rats. In conclusion, the hypotensive efficacy of nebivolol is significantly enhanced in L-NAME rats compared with normotensive animals, which is most likely due to a greater reduction in vascular sympathetic activity. Nebivolol markedly attenuated short-term BPV in both experimental groups, suggesting that β-blockers with additional pharmacological actions provide beneficial cardiovascular effects by controlling high BP and its short-term variability.
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Keywords: blood pressure variability; enantioselective pharmacokinetics; L-NAME hypertension; nebivolol; spectral analysis

INTRODUCTION

Nebivolol is a third-generation β-blocker that possesses important ancillary properties besides selective antagonism of the β_1-adrenoceptor.[1] Nebivolol induces the activation of nitric oxide (NO) synthase and preserves its function because of its antioxidant properties of enhancing NO bioavailability.[1] As impaired NO synthase activity and reduced NO bioavailability are common initial mechanisms of cardiovascular dysfunction, nebivolol exerts additional pharmacological effects including vasodilatation, antiproliferation and cardioprotection, possibly resulting in more effective organ protection in comparison with conventional β-blockers.[1,2]

Although the blood pressure (BP)-lowering effect of nebivolol and its mechanism of action have been extensively studied in previous works, little is known about the effect of this third-generation β−blocker on BP variability (BPV), an independent risk factor for the incidence of cardiovascular events.[3–5] As enhanced BPV has been associated with target organ damage in both normotensive and hypertensive subjects, antihypertensive agents need not only control mean arterial pressure (MAP) but also attenuate its short- and long-term variability.[6,7]

Considering the NO-dependent vasodilatory properties of nebivolol, the hypertensive stage induced by chronic L-NAME administration represents an attractive experimental model to discern the relative contributions of the ancillary properties of nebivolol to its pharmacological profile. Concomitant administration of nebivolol with L-NAME has been shown to partially prevent the hypertensive stage induced by NO synthase inhibition.[8] Nevertheless, to the best our knowledge, the pharmacokinetics and acute effects of nebivolol administration on BP and its short-term variability have not been previously studied in L-NAME hypertensive animals.

In accordance, the aim of this work was the comprehensive assessment of the pharmacokinetics and the *in vivo* cardiovascular

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properties of nebivolol in L-NAME hypertensive rats, including the effects on heart rate (HR), BP, regulation and its action on beat-to-beat and short-term BPV.

METHODS

Animals

Male Wistar rats (3 months old, 220–250 g) were purchased from the School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina. Animal experiments were performed in accordance with the published Guide for the Care and Use of Laboratory Animals (NIH, eighth edition, 2011) and were approved by the local Scientific and Technology Ethics Committee at the University of Buenos Aires. All efforts were made to minimize animal suffering and to reduce the number of animals used. The rats were housed six per cage and randomly divided into two groups. Control rats ($n=18$) were given tap water to drink for 2 weeks. L-NAME hypertensive rats ($n=18$) were given L-NAME solution at a concentration of 0.4 mg ml^{-1} (40 mg kg^{-1} per day) to drink for 2 weeks. The dose of L-NAME was not adjusted during the treatment period to maintain the weight of the rats. The dose of L-NAME was selected according to our and others' works.[9,10]

Preparation of nebivolol formulation

Nebivolol is practically insoluble in water, and therefore, a special formula was prepared to allow i.v. administration of the drug at doses of 3 and 10 mg kg^{-1}. The formula of the nebivolol solution consisted of 0.3 or 1.0% (w/v) nebivolol (a gift from Laboratorios Raffo, Buenos Aires, Argentina), 0.5% (w/v) polyvinylpyrrolidone, 40% (v/v) propylene glycol, 10% (v/v) glycerine and purified water.

Experimental design

The animals were anesthetized with ether, and the left carotid arteries and left femoral veins were cannulated with a polyethylene cannulae containing a heparinized saline solution (25 U ml^{-1}). The cannulae were tunneled under the skin and externalized at the back of the neck. The experiments were performed in freely moving animals 24 h after cannulae placement. A recovery period of 24 h has been found to be adequate for the evaluation of drug effects on BPV in conscious rats.

On the day of the experiment, the arterial cannulae were connected to a Spectramed P23XL pressure transducer (Spectramed, Oxnard, CA, USA) coupled to a Grass 79D polygraph (Grass Instrument, Quincy, MA, USA). The polygraph was connected to a digital converter adaptor unit (Polyview, PVA 1, Grass-Astro Med, West Warwick, RI, USA), and the recordings were stored and analyzed using a software program (Polyview 2.3 Astro-Med). Basal MAP and HR were estimated during an interval of 60 min before drug administration. MAP was calculated as the sum of the diastolic pressure and one-third of the pulse pressure. HR was estimated tachographically by counting the pulsatile waves of arterial pressure recording.

Nebivolol 3 mg kg^{-1} ($n=6$ of each group), nebivolol 10 mg kg^{-1} ($n=6$ of each group) or vehicle ($n=6$ of each group) were injected i.v. over 30 s. After nebivolol administration, MAP and HR were continuously recorded and blood samples (100 µl) were collected from the arterial cannulae at the following time points: 5, 10, 15, 30, 60, 90, 120 and 180 min. To reduce the effects attributable to circadian alterations, all experiments were conducted between 1300 and 1900 hours.

Analytical determination of nebivolol

Arterial blood samples (100 µl) collected in polypropylene microcentrifuge tubes containing 5 µl of heparinized solution were centrifuged at 3000 *g* for 10 min under controlled temperature (4 °C). It is important to mention that blood sampling could alter the pharmacokinetic and pharmacodynamic behavior of antihypertensive drugs due to fluid loss. Nevertheless, in our experimental protocol we only extracted approximately 800 µl of blood during a 3-h period for estimation of plasma concentrations of nebivolol. This volume is significantly lower than the recommended maximal volume of blood to be removed (3.5 ml) in a rat weighing 250 g,[11] and therefore, it could be suggested that blood loss during our experimental protocol did not affect the pharmacokinetic and pharmacodynamic properties of nebivolol.

Plasma supernatant (30 µl) was carefully separated, and nebivolol was extracted using a liquid procedure. Briefly, an aliquot of internal standard (2 µg ml^{-1} propranolol in methanol), 0.50 M sodium bicarbonate (50 µl) and dichloromethane (1 ml) were added to a 30 µl of plasma sample. The mixture was vortexed for 2 min and centrifuged at 2000 r.p.m. for 10 min. The organic layer was transferred into a conical tube and evaporated under nitrogen gas. The dry extract was reconstituted with 100 µl of mobile phase and injected into the chromatographic system. A stock solution of racemic nebivolol standard in blank plasma was used for the quantification of *d*- and *l*-nebivolol levels in the plasma samples.

The levels of *d*- and *l*-nebivolol in plasma samples were measured by normal phase liquid chromatography with fluorescence detection using a chiral column (Chirex (S)-ICA and (R)-NEA, Phenomenex, Torrance, CA, USA) and a fluorescence detector (FL-3000, Thermo Finnigan, Les Ullis, France). The excitation and emission wavelengths used were 282 and 318 nm, respectively. Optimal composition of the mobile phase was achieved by a mixture of hexane, dichloromethane, ethanol and trifluoroacetic acid (65:35:5:0.2). The retention times of *d*- and *l*-nebivolol in our chromatographic conditions were 5.7 ± 0.4 and 7.2 ± 0.5 min, respectively. The coefficient of variation of the chromatographic method was $<5\%$, and the limit of quantification of *d*- and *l*-nebivolol was 200 ng ml^{-1}. The intraday and interday coefficients of variation were 4.8 and 4.6, respectively.

Estimation of BPV

BPV was continuously estimated by the determination of the s.d. and spectral analysis of the 3-min periods of BP recordings obtained from baseline and during regular times after nebivolol administration when the quality of the arterial BP signal was visually considered to be satisfactory. According to our and other works, spectral analysis of the data was performed using the Fast Fourier Transform algorithm with a Hamming window (Polyview 2.3 Astro-Med).[9,12] Spectral densities in the very low-frequency (VLF) range (0.1–0.2 Hz), in the low-frequency (LF) range (0.2–0.7 Hz) and in the high-frequency (HF) range (0.7–2.5 Hz) were calculated from the baseline recordings and after nebivolol racemate administration.[9,12] Although LF variability is affected by sympathetic modulation of vascular tone, we used the LF/HF ratio as an index of vascular sympathetic activity. The normalization procedure tends to minimize the effect of the changes in total power on the absolute values of LF variability.[12,13]

Pharmacokinetic analysis

The pharmacokinetics of total plasma *d*- and *l*-nebivolol concentrations were estimated by compartmental analysis by applying a two-compartment, first-order elimination model. Non-linear least-squares regression analysis was performed using the TOPFIT program (version 2.0, Dr Karl Thomae Gmbh, Schering AG, Gödecke AG, Germany), which uses a cyclic three-stage optimization routine (one-dimensional direct search; vectorial direct search/Hooke–Jeeves modified; Gauss–Newton/Marquadt modified). Pharmacokinetic parameters were estimated using both micro and macroconstants. No weighing scheme was used during pharmacokinetic parameter estimation. The area under the curve of the nebivolol levels *vs.* time (from 0 to infinity) was calculated using the linear trapezoidal rule. Clearance and steady-state volume of distribution were estimated by standard methods.[14]

Statistical analysis

The normal distribution of the data and the variables of the study were verified using the Kolmogorov–Smirnov test. Data were expressed as the means ± s.e.m. The basal values of cardiovascular parameters in L-NAME and control rats were compared using Student's *t*-test. The statistical analysis of nebivolol's effect on MAP, HR, s.d. and the LF/HF ratio was performed by two-way analysis of variance and Bonferroni's test as the *post-hoc* test. The pharmacokinetic parameters were log transformed for statistical analysis to reduce the heterogeneity of the variance and were further compared by two-way analysis of variance and Bonferroni's test as the *post-hoc* test. Statistical tests were

performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as $P<0.05$.

RESULTS

The baseline values of the cardiovascular parameters and the BPV of L-NAME hypertensive rats and control animals are shown in Table 1. MAP and short-term BPV were significantly increased in L-NAME rats compared with normotensive animals (Table 1). No differences were found in the HR baseline values between both experimental groups. Spectral analysis of BP recordings showed an increase in the beat-to-beat BPV in the VLF and LF domain in hypertensive L-NAME rats when compared with the control animals, without significant changes in HF variability (Table 1). Vascular sympathetic activity was significantly enhanced in L-NAME rats considering the fact that the LF/HF ratio was increased by 29.8% in the hypertensive group with respect to the normotensive control rats (Table 1).

Nebivolol pharmacokinetics

Figure 1 shows the temporal course of *d*- and *l*-nebivolol plasma concentrations in control ($n=12$) and L-NAME rats ($n=12$) after i.v. administration. A biexponential decay of plasma nebivolol levels was found in all experiments compatible with a pharmacokinetic two-compartment model after 3 and 10 mg kg^{-1} of nebivolol (Figure 1). The plasma levels of *l*-nebivolol were significantly lower compared with the *d*-enantiomer in both experimental groups, demonstrating the existence of an enantioselective pharmacokinetic behavior of nebivolol racemate (Figure 1). A comparison of the pharmacokinetic parameters showed a greater volume of distribution and clearance of *l*-nebivolol with regards to *d*-nebivolol in both L-NAME rats and control animals (Table 2). Consequently, the area under the curve and C_{max} of *l*-nebivolol were significantly lower in comparison with the *d*-enantiomer. The hypertensive stage induced by chronic L-NAME administration seems to not affect nebivolol enantioselective pharmacokinetics. No differences were found when comparing the major pharmacokinetic parameters of *d*- and *l*-nebivolol in both L-NAME hypertensive rats and normotensive control animals (Table 2).

Effects of i.v. administration of nebivolol on HR

Figure 2 shows the temporal course of HR changes in L-NAME and control rats after vehicle or nebivolol i.v. administration at doses of 3 or 10 mg kg^{-1}. Although vehicle administration did not modify HR in either experimental group, nebivolol racemate induced a bradycardic response at both dose levels in L-NAME and control rats with complete recovery of baseline HR values during follow-up. Although the maximal chronotropic response to nebivolol administration did not differ between both experimental groups, HR returned to baseline values earlier in the L-NAME group compared with the control rats after administration of the lower dose (Figure 2).

Table 1 Baseline cardiovascular parameters of control and L-NAME hypertensive rats

Parameter	*Control rats* (n = *18*)	*L-NAME rats* (n = *18*)
Cardiovascular parameters		
MAP (mm Hg)	105 ± 3	132 ± 2*
HR (b.p.m.)	348 ± 8	341 ± 6
Blood pressure variability		
s.d. (mm Hg)	3.97 ± 0.26	5.27 ± 0.26*
VLF variability (mm Hg^2)	11.7 ± 1.0	22.5 ± 1.4*
LF variability (mm Hg^2)	8.9 ± 0.7	15.3 ± 1.1*
HF variability (mm Hg^2)	2.5 ± 0.3	3.2 ± 0.3
LF/HF ratio	3.79 ± 0.21	4.92 ± 0.28*

Abbreviations: HF, high frequency; HR, heart rate; MAP, mean arterial pressure; LF, low frequency; VLF, very low frequency.
*$P<0.05$ *vs.* control rats by Student's *t*-test.

Effects of i.v. administration of nebivolol of on BP

Hypotensive response to i.v. administration of nebivolol (3 or 10 mg kg^{-1}) is depicted in Figure 3. No changes in MAP were found in either of the experimental groups after vehicle administration. The BP-lowering effects of nebivolol showed a biphasic pattern in both normotensive and L-NAME hypertensive rats (Figure 3). After 3 and 10 mg kg^{-1} i.v. administration, the early reduction of MAP values was significantly greater in L-NAME hypertensive animals compared with the control rats (Figure 3). Conversely, no differences were found in the magnitude of late BP reduction induced by the administration of nebivolol between both experimental groups (Figure 3).

Effects of i.v. nebivolol administration on beat-to-beat and short-term BPV

Nebivolol i.v. administration markedly reduced short-term BPV in both normotensive and L-NAME hypertensive rats as evidenced by the sustained reduction in the s.d. of the BP after administration of 3 or 10 mg kg^{-1} of the drug (Figure 4). Although no differences were

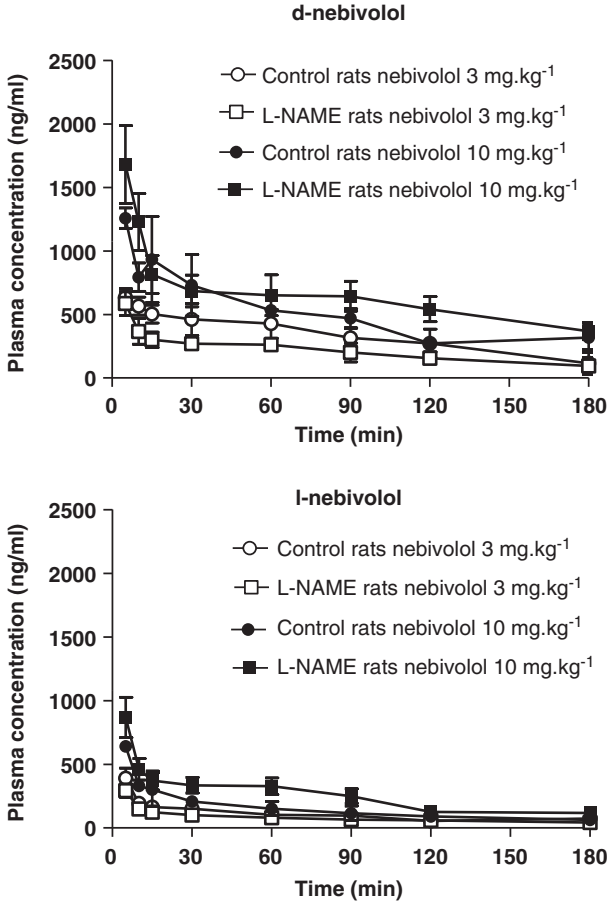

Figure 1 Mean plasma concentration values of *d*- and *l*-nebivolol *vs.* time in control rats (circles) and L-NAME hypertensive animals (squares) after administration of 3 mg kg^{-1} (open symbols) or 10 mg kg^{-1} (black symbols) of the drug. Each point shows the mean ± s.e.m. of six rats.

Table 2 Pharmacokinetic parameters of total *d*- and *l*-nebivolol plasma levels obtained from arterial blood samples in control and L-NAME rats after i.v. administration of drug (3 and 10 mg kg^{-1}); AUC, α, β, Cl, Vd_{ss}, C_{max} and AIC

Enantiomer	*D-nebivolol*				*L-nebivolol*			
Experimental group	*Control rats (n = 12)*		*L-NAME rats (n = 12)*		*Control rats (n = 12)*		*L-NAME rats (n = 12)*	
Dose	3 mg kg^{-1}	10 mg kg^{-1}	3 mg kg^{-1}	10 mg kg^{-1}	3 mg kg^{-1}	10 mg kg^{-1}	3 mg kg^{-1}	10 mg kg^{-1}
α (h^{-1})	8.1 ± 2.7	11.6 ± 0.1	12.8 ± 0.8	8.9 ± 1.8	14.9 ± 0.1	12.0 ± 1.7	11.4 ± 1.8	10.8 ± 1.7
β (h^{-1})	0.36 ± 0.11	0.35 ± 0.07	0.35 ± 0.04	0.33 ± 0.08	0.43 ± 0.06	0.66 ± 0.08[a]	0.49 ± 0.11	0.47 ± 0.14
Vd_{ss} (l)	3.8 ± 0.3	5.4 ± 1.8	4.2 ± 1.0	5.5 ± 1.0	8.3 ± 0.8	13.9 ± 2.0[a]	9.7. ± 2.1	12.7 ± 2.3[a]
Cl (ml min^{-1})	23.4 ± 6.2	32.2 ± 3.5	22.3 ± 5.7	28.3 ± 2.6	62.9 ± 5.6[a]	82 ± 11.8[a]	71.8 ± 18.1[a]	74.4 ± 15.2[a]
C_{max} (ng ml^{-1})	1522 ± 534	2620 ± 154[b]	1350 ± 238	2792 ± 306[b]	418 ± 41	1239 ± 391[a,b]	594 ± 280	1356 ± 258[a,b]
$AUC_{0^{-}\infty}$ (ng ml h^{-1})	808 ± 70	2120 ± 365[b]	1044 ± 257	2593 ± 364[b]	596 ± 134	1244 ± 270[a,b]	552 ± 162	1409 ± 197[a,b]
r^2	0.993 (0.988–0.999)	0.991 (0.981–0.998)	0.998 (0.996–0.999)	0.994 (0.984–0.999)	0.998 (0.998–0.999)	0.996 (0.994–0.997)	0.997 (0.995–0.999)	0.992 (0.967–0.999)
AIC	59.9 (46.6–84.7)	81.8 (63.1–103.5)	53.8 (28.9–71.3)	89.9 (76.8–109)	46.8 (33.2–66.0)	64.7 (51.0–76.8)	44.8 (32.6–54.5)	68.5 (31.6–98.2)

Abbreviations: α, constant of distribution; AIC, Akaike Information Criterion; ANOVA, analysis of variance; AUC, area under the curve; β, constant of elimination; Cl, clearance; C_{max}, extrapolated maximal concentration; Vd_{ss}, steady-state volume of distribution.
Data are expressed as mean ± s.e.m. Goodness of fit indicators are expressed as mean (range).
[a]$P<0.05$ *vs. d*-nebivolol by two-way ANOVA followed by Bonferroni post-test.
[b]$P<0.05$ *vs.* 3 mg kg^{-1} by two-way ANOVA followed by Bonferroni post-test.

Figure 2 Time course of changes in heart rate (Δ heart rate (HR), percentage of baseline values), after i.v. administration of nebivolol (open symbols) or vehicle (black symbols) in normotensive control rats (circles) and L-NAME hypertensive animals (squares). Each point shows the mean ± s.e.m. of six rats. *$P<0.05$ *vs.* control rats by two-way analysis of variance (ANOVA) followed by Bonferroni post-test.

found in the magnitude of s.d. attenuation induced by 3 mg kg^{-1} nebivolol between both experimental groups, a slightly greater response to nebivolol was observed in the L-NAME rats when compared with the control animals after administration of the higher dose (Figure 4).

Beat-to-beat BPV was also significantly reduced by nebivolol administration in both experimental groups. Vehicle administration did not modify BPV at the three domains (VLF, LF and HF) in either of the experimental groups (data not shown). I.v. administration of nebivolol induced a greater attenuation of VLF variability in the L-NAME hypertensive rats (% of baseline value: 3 mg kg^{-1}: −34.1 ± 7.9%, $n=6$; 10 mg kg^{-1}: –47.9 ± 7.3%, $n=6$; $P<0.05$ *vs.* control rats) when compared with normotensive animals (% of baseline value: 3 mg kg^{-1}: −7.0 ± 7.9%, $n=6$; 10 mg kg^{-1}: −20.8 ± 7.8%, $n=6$). Conversely, a reduction of the beat-to-beat BPV at LF was not significantly different between normotensive (% of baseline value: 3 mg kg^{-1}: −29.4 ± 6.3%, $n=6$; 10 mg kg^{-1}: −26.9 ± 5.3%, $n=6$) and L-NAME hypertensive rats (% of baseline value: 3 mg kg^{-1}: −41.9 ± 6.3%, $n=6$; 10 mg kg^{-1}: −44.6 ± 6.8%, $n=6$). In addition, changes in HF BPV were comparable in both the L-NAME rats (% of baseline value: 3 mg kg^{-1}: −30.6 ± 2.7%, $n=6$; 10 mg kg^{-1}: −18.2 ± 4.4%, $n=6$) and the normotensive control

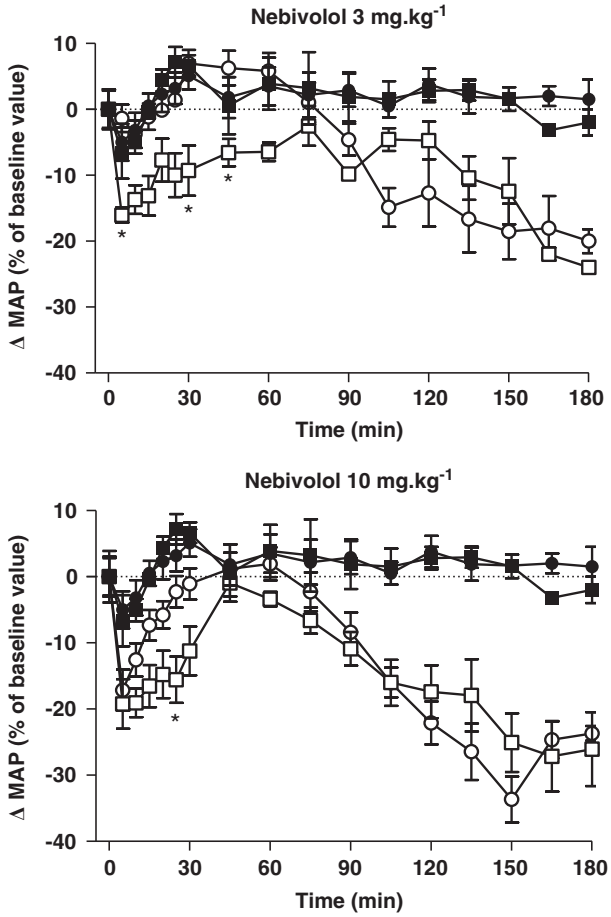

Figure 3 Time course of changes in mean arterial pressure (Δ mean arterial pressure (MAP), percentage of baseline values), after i.v. administration of nebivolol (open symbols) or vehicle (black symbols) in normotensive control rats (circles) and L-NAME hypertensive animals (squares). Each point shows the mean ± s.e.m. of six rats. $^*P<0.05$ *vs.* control rats by two-way analysis of variance (ANOVA) followed by Bonferroni post-test.

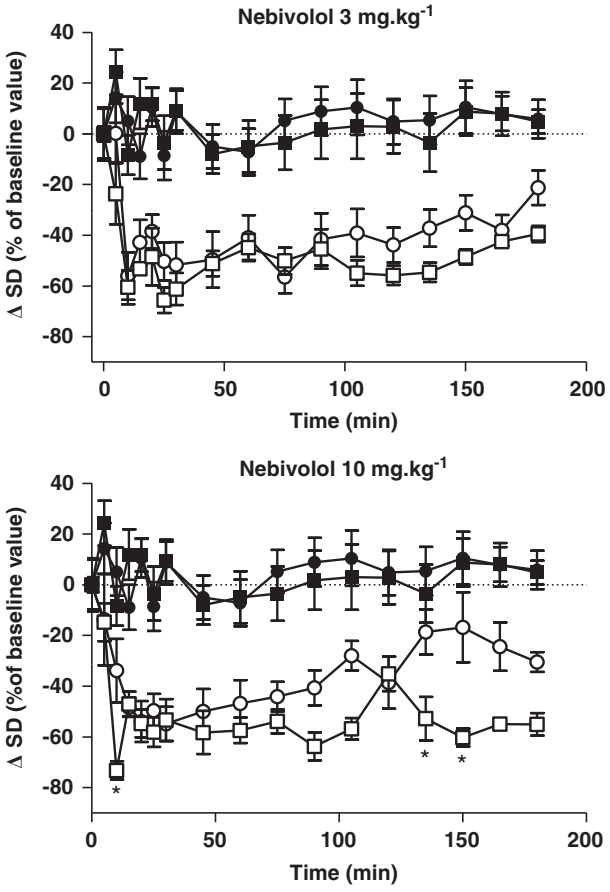

Figure 4 Change in short-term blood pressure variability, expressed as percent of baseline s.d. value, after i.v. administration of nebivolol (open symbols) or vehicle (black symbols) in normotensive control rats (circles) and L-NAME hypertensive animals (squares). Each point shows the mean ± s.e.m. of six rats. $^*P<0.05$ *vs.* control rats by two-way analysis of variance (ANOVA) followed by Bonferroni post-test.

animals (% of baseline value: 3 mg kg^{-1}: $-26.2 \pm 7.4\%$, $n=6$; 10 mg kg^{-1}: $-13.3 \pm 5.4\%$, $n=6$).

The effects of nebivolol on the LF/HF ratio, a marker of vascular sympathetic activity, were also assessed in the control and L-NAME rats after administration of vehicle or nebivolol 3 or 10 mg kg^{-1}. Although vehicle did not modify the LF/HF ratio in either of the experimental groups, nebivolol induced a significant reduction in this parameter in normotensive and L-NAME animals at both dose levels (Figure 5). Moreover, L-NAME hypertensive rats showed a greater reduction in the LF/HF ratio when compared with the control group at both dose levels (Figure 5).

DISCUSSION

This study yielded several findings regarding the pharmacokinetic and pharmacodynamic properties of nebivolol in L-NAME hypertensive rats. The enantioselective pharmacokinetics of nebivolol were evidenced by the fact that *l*-nebivolol exhibits a greater volume of distribution and clearance than *d*-enantiomer in both experimental groups. In addition, the hypertensive stage induced by chronic administration of L-NAME seems not to affect the pharmacokinetic properties of nebivolol. Although L-NAME administration interferes with the NO system, acute nebivolol administration induced a greater early hypotensive response in the hypertensive group in comparison with the control normotensive animals. Finally, i.v. administration of nebivolol markedly ameliorated short-term BPV in both L-NAME hypertensive and control rats.

L-NAME administration in tap water at a dose of 40 mg kg^{-1} for 2 weeks induced a significant increase in MAP without changes in HR. The magnitude of the BP elevation achieved with this dose level in this study is similar to previously reported results by us and other authors using the same dose.[9,10] Moreover, an increase in 30–40 mm Hg in BP has also been obtained after administration of L-NAME at a dose of 10, 15 or 60 mg kg^{-1} per day.[8,15,16] Therefore, it could be suggested that the hypertensive stage induced by chronic administration of L-NAME reaches a maximal response with 10–15 mg kg^{-1} per day, and further dose increments do not contribute to larger elevations in BP.

Nebivolol pharmacokinetics were studied 24 h after arterial cannulation. It has been demonstrated that surgical implantation of cannulae 24 h before the experiment induced an increase in α_1-glycoprotein.[17] As nebivolol binds predominantly to serum albumin,[2] it seems unlikely that an increase in α_1-glycoprotein due to cannulae implantation would affect drug pharmacokinetics in our experimental conditions. Nebivolol pharmacokinetics have been studied previously in human subjects. This β-blocker undergoes high hepatic extraction and is

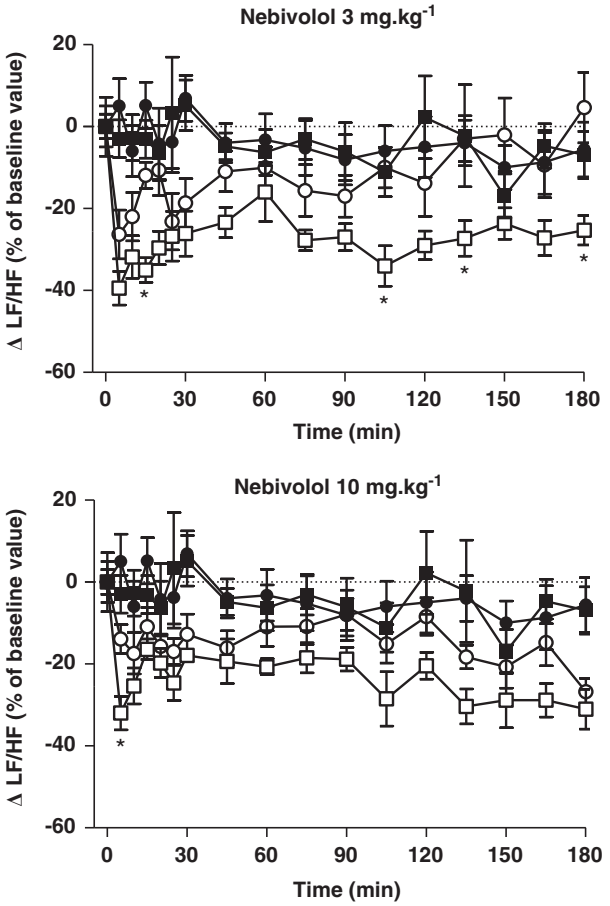

Figure 5 Time course of changes in normalized low-frequency (LF) variability (LF/high frequency (HF) ratio), expressed as % of baseline values, after i.v. administration of nebivolol (open symbols) or vehicle (black symbols) in normotensive control rats (circles) and L-NAME hypertensive animals (squares). Each point shows the mean ± s.e.m. of six rats. $^{*}P<0.05$ *vs.* control rats by two-way analysis of variance (ANOVA) followed by Bonferroni post-test.

extensively metabolized by the cytochrome P450 2D6 to its hydroxylated active metabolite.[2] In addition, nebivolol exhibits stereoselective pharmacokinetic properties, considering that the peak and trough plasma concentrations of *d*-nebivolol were greater than those of the *l*-enantiomer after single and multiple administrations in patients.[18] The enantioselective pharmacokinetic pattern of nebivolol was also evidenced in experimental models of hypertension. Plasma levels of *l*-nebivolol were significantly lower than the levels of *d*-nebivolol in both spontaneously hypertensive and Wistar Kyoto animals.[19] In accordance with these previous findings, in this study, nebivolol showed enantioselective pharmacokinetic properties in L-NAME and control rats supported by the fact that the clearance and volume of distribution of *l*-nebivolol were significantly greater than those of *d*-nebivolol in both experimental groups. In addition, pharmacokinetic analysis demonstrated that the main pharmacokinetic parameters of *l*- and *d*-nebivolol were similar between both experimental groups, suggesting that the hypertensive stage induced by chronic administration of L-NAME does not affect the pharmacokinetic behavior of nebivolol. These findings contrast with our previous results obtained in spontaneously hypertensive rats, which showed a reduced clearance of nebivolol in this experimental model of hypertension with regard to normotensive Wistar Kyoto animals.[19]

Nebivolol i.v. administration induced a dose-dependent reduction in BP in L-NAME and control rats that was characterized by an early reduction in MAP values followed by a sustained BP-lowering effect during the complete follow up. Sustained hypotensive response to nebivolol has been previously described by us and other authors.[19,20] This pharmacodynamic pattern of nebivolol greatly contrasts with other β-blockers, such as metoprolol and carvedilol, which induce a fast decrease in MAP followed by an early recovery of baseline values.[14,21,22] Different mechanisms could explain the long-acting hypotensive response induced by nebivolol, including the slow rate of dissociation from its receptor and the generation of active metabolites that perpetuate the hypotensive effect of nebivolol.[23,24]

A comparison of nebivolol effects on BP in both experimental groups demonstrates an enhanced early hypotensive response to the β-blocker in L-NAME rats with regard to the normotensive control animals. Conversely, the late BP lowering of nebivolol was not increased in the hypertensive group compared with control rats. As treatment with L-NAME has been shown to partially prevent the cardiovascular actions of nebivolol,[8] the enhanced BP-lowering effect of nebivolol in L-NAME rats seems to be paradoxical. Nevertheless, it is well known that the hypertensive state in chronically treated L-NAME hypertensive animals is not only induced by the abolition of the NO system but also by overactivity of the renin–angiotensin system and the sympathetic nervous system.[25–28] Therefore, the increased hypotensive response to nebivolol in L-NAME hypertensive rats could be explained by its ability to reduce renin–angiotensin and sympathetic overactivity. Previous studies have demonstrated that treatment with nebivolol reduces plasma renin and angiotensin II levels in spontaneously hypertensive rats.[29,30] Moreover, nebivolol prevents the development of the arterial hypertension induced by chronic NO deficiency partially due to the inhibition of the renin–angiotensin system.[8] Sympatholytic actions of nebivolol have been previously described both in experimental animals and healthy subjects.[31,32]

To support our hypothesis, in this study we have assessed the effects of nebivolol on the beat-to-beat BPV by means of spectral analysis of BP recordings. Identification of the frequency components of BPV by power spectral analysis can potentially provide information about mechanisms involved in BP regulation.[33] In fact, although the renin–angiotensin system and endothelial-derived NO modulate BPV at the VLF component, LF variability is controlled by the sympathetic modulation of vascular tone and endothelial-derived NO.[33] Variability in the HF domain is mainly influenced by changes in cardiac output.[34] Finally, normalized LF (LF/HF ratio) has been validated as a marker of sympathetic vascular activity in preclinical and clinical studies.[13,35] Analysis of baseline values of beat-to-beat BPV demonstrated a greater VLF and LF/HF ratio in L-NAME rats compared with control normotensive animals, suggesting important contributions of the renin–angiotensin system and the vascular sympathetic system in BP regulation in hypertensive rats. Moreover, nebivolol administration induced a greater attenuation of BPV at the VLF domain in L-NAME rats than in control animals, supporting an enhanced action of nebivolol on the renin–angiotensin system of hypertensive rats. In addition, i.v. administration of nebivolol (3 or 10 mg kg^{-1}) reduced the LF/HF ratio to a greater extent in L-NAME rats compared with normotensive animals, suggesting that attenuation of vascular sympathetic activity could also contribute to the enhanced hypotensive response to nebivolol in L-NAME rats.

This study has also assessed the acute effects of nebivolol on short-term BPV estimated by the s.d. of the BP recording in both experimental groups. Recent findings have highlighted the prognostic

value of BPV on cardiovascular morbidity and mortality in both normotensive and hypertensive subjects.[5] Excessive fluctuation of BP contributes to the progression of organ damage and in triggering vascular events, particularly stroke.[5] Moreover, increased BPV has been associated with left ventricular hypertrophy in normotensive subjects.[6] Considering the contribution of BPV to the development of cardiovascular events, reduction of this parameter should be considered as a possible new target in cardiovascular medicine.[7]

Post-hoc analysis of clinical trials has demonstrated that antihypertensive drugs differ in their ability to reduce BPV, even within the same pharmacological group.[36] Treatment with conventional β-blockers has been associated with an increase in BPV in hypertensive patients, and a subanalysis of the ASCOT-BPLA trial results showed that amlodipine exerts greater protection against cerebrovascular events in hypertensive patients than atenolol because of its ability to reduce short-term and long-term BPV.[37] Nevertheless, the extrapolation of these findings to third-generation β-blockers, such as nebivolol, seems not to be adequate considering that β-blockers greatly differ in their pharmacokinetic and pharmacodynamic properties.[38,39] Moreover, a recent systematic review has found that variability in systolic BP is increased more by non-selective β-blockers than by selective β1-adrenergic antagonists.[40] In fact, in a recent study we found that i.v. administration of a single dose of nebivolol or carvedilol greatly reduce short-term BPV in sinoaortic denervated (SAD) rats.[41] Conversely, cardioselective blockade of β_1-adrenoceptor with atenolol induces only minor beneficial effects on BP fluctuations in SAD animals.[41]

Compared with normotensive control animals, chronic intake of L-NAME significantly increased baseline s.d. values confirming that short-term BPV increases with BP levels. In agreement with our findings, an enhancement in the s.d. of BP recordings has been previously found in L-NAME hypertensive rats by other authors.[42,43] Nebivolol i.v. administration dramatically reduced the s.d. of BP recordings in both experimental groups, suggesting that this third-generation β-blocker does not only effectively control the hypertensive stage induced by L-NAME administration but also attenuates short-term BPV. Moreover, the reduction in short-term BPV induced by nebivolol seems not to be directly related to its BP-lowering action. Meanwhile, the reduction of BP induced by nebivolol showed a biphasic pattern in both experimental groups, and a sustained reduction in short-term BPV was observed during the entire experiment. Nebivolol i.v. administration induced a greater attenuation of the s.d. of the BP recordings in L-NAME hypertensive rats compared with normotensive control animals. Previous findings suggested that the sympathetic nervous system and the renin–angiotensin system are major determinants of the increased short-term variability of BPV in L-NAME hypertensive rats.[43,44] Therefore, the enhanced ability of nebivolol to control short-term BPV in L-NAME rats could be explained, in part, by its greater vascular sympatholytic activity in the hypertensive animals.

In conclusion, the findings of this study provide new insights in the pharmacokinetic and cardiovascular properties of the third-generation β-blocker nebivolol in experimental models of hypertension. The enantioselective pharmacokinetic properties of nebivolol were not affected by the hypertensive stage induced by L-NAME administration. Acute administration of nebivolol exerts a sustained and dose-dependent hypotensive response in both normotensive control and L-NAME hypertensive animals. Moreover, BP-lowering efficacy is significantly enhanced in L-NAME rats compared with normotensive animals, which is most likely due to a greater reduction in the activity of the renin–angiotensin system and vascular sympathetic activity. Nebivolol markedly attenuated short-term BPV in both experimental groups, suggesting that β-blockers with additional pharmacological actions, such as nebivolol, provide beneficial cardiovascular effects by both controlling high BP and its short-term variability.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Vanhoutte PM, Gao Y. Beta blockers, nitric oxide, and cardiovascular disease. *Curr Opin Pharmacol* 2013; **13**: 265–273.
2 Gao Y, Vanhoutte PM. Nebivolol: an endothelium-friendly selective β1-adrenoceptor blocker. *J Cardiovasc Pharmacol* 2012; **59**: 16–21.
3 Parati G, Ochoa JE, Lombardi C, Bilo G. Assessment and management of blood-pressure variability. *Nat Rev Cardiol* 2013; **10**: 143–155.
4 Su DF, Miao CY. Blood pressure variability and organ damage. *Clin Exp Pharmacol Physiol* 2001; **28**: 709–715.
5 Rothwell PM. Does blood pressure variability modulate cardiovascular risk? *Curr Hypertens Rep* 2011; **13**: 177–186.
6 Schutte AE, Schutte R, Huisman HW, van Rooyen JM, Fourie CM, Malan NT, Malan L. Blood pressure variability is significantly associated with ECG left ventricular mass in normotensive Africans: the SABPA Study. *Hypertens Res* 2011; **34**: 1127–1134.
7 Schillaci G, Pucci G, Parati G. Blood pressure variability: an additional target for antihypertensive treatment? *Hypertension* 2001; **58**: 133–135.
8 Fortepiani LA, Ortíz MC, Atucha NM, García-Estañ J. Nebivolol ameliorates nitric oxide deficient hypertension. *ScientificWorldJournal* 2002; **2**: 1676–1684.
9 Di Verniero CA, Bertera F, Buontempo F, Bernabeu E, Chiappetta D, Mayer MA, Bramuglia GF, Taira CA, Höcht C. Enantioselective pharmacokinetic-pharmacodynamic modelling of carvedilol in a N-nitro-l-arginine methyl ester rat model of secondary hypertension. *J Pharm Pharmacol* 2010; **62**: 890–900.
10 Bernátová I, Pechánová O, Pelouch V, Simko F. Regression of chronic L-NAME-treatment-induced left ventricular hypertrophy: effect of captopril. *J Mol Cell Cardiol* 2000; **32**: 177–185.
11 Aimone LD. Overview of pharmacokinetics. *Curr Protoc Pharmacol* 2005; **7**: 1–26.
12 Pladys P, Lahaie I, Cambonie G, Thibault G, Le NL, Abran D, Nuyt AM. Role of brain and peripheral angiotensin II in hypertension and altered arterial baroreflex programmed during fetal life in rat. *Pediatr Res* 2004; **55**: 1042–1049.
13 Souza HC, Martins-Pinge MC, Dias da Silva VJ, Borghi-Silva A, Gastaldi AC, Blanco JH, Tezini GC. Heart rate and arterial pressure variability in the experimental renovascular hypertension model in rats. *Auton Neurosci* 2008; **139**: 38–45.
14 Gibaldi M, Perrier D. *Pharmacokinetics*, 2nd edn. Marcel Dekker, New York, 1982.
15 Moningka NC, Tsarova T, Sasser JM, Baylis C. Protective actions of nebivolol on chronic nitric oxide synthase inhibition-induced hypertension and chronic kidney disease in the rat: a comparison with angiotensin II receptor blockade. *Nephrol Dial Transplant* 2012; **27**: 913–920.
16 Ribeiro MO, Antunes E, de Nucci G, Lovisolo SM, Zatz R. Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. *Hypertension* 1992; **20**: 298–303.
17 Terao N, Shen DD. Alterations in serum protein binding and pharmacokinetics of l-propranolol in the rat elicited by the presence of an indwelling venous catheter. *J Pharmacol Exp Ther* 1983; **227**: 369–375.
18 Van Peer A, Snoeck E, Woestenborghs R, Van de Velde V, Mannens G, Meuldermans W, Heykants J. Clinical pharmacokinetics of nebivolol. *Drug Invest* 1992; **3**: 25–30.
19 Bertera FM, Del Mauro JS, Polizio AH, Chiappetta D, Taira CA, Höcht C. Effect of nebivolol on beat-to-beat and short-term blood pressure variability in spontaneously hypertensive rats. *Naunyn Schmiedebergs Arch Pharmacol* 2012; **385**: 833–843.
20 Van de Water A, Janssens W, Van Neuten J, Xhonneux R, De Cree J, Verhaegen H, Reneman RS, Janssen PA. Pharmacological and hemodynamic profile of nebivolol, a chemically novel, potent, and selective beta 1-adrenergic antagonist. *J Cardiovasc Pharmacol* 1988; **11**: 552–563.
21 Höcht C, Di Verniero C, Opezzo JA, Bramuglia GF, Taira CA. Pharmacokinetic-pharmacodynamic (PK-PD) modeling of cardiovascular effects of metoprolol in spontaneously hypertensive rats: a microdialysis study. *Naunyn Schmiedebergs Arch Pharmacol* 2006; **373**: 310–318.
22 Bertera FM, Del Mauro JS, Chiappetta D, Polizio AH, Buontempo F, Taira CA, Höcht C. Enantioselective pharmacokinetic and pharmacodynamic properties of carvedilol in spontaneously hypertensive rats: focus on blood pressure variability. *Naunyn Schmiedebergs Arch Pharmacol* 2012; **385**: 325–335.
23 Kuroedov A, Cosentino F, Lüscher TF. Pharmacological mechanisms of clinically favorable properties of a selective beta1-adrenoceptor antagonist, nebivolol. *Cardiovasc Drug Rev* 2004; **22**: 155–168.

24 Himmelmann A, Hedner T, Snoeck E, Lundgren B, Hedner J. Haemodynamic effects and pharmacokinetics of oral d- and l-nebivolol in hypertensive patients. *Eur J Clin Pharmacol* 1996; **51**: 259–264.
25 Biancardi VC, Bergamaschi CT, Lopes OU, Campos RR. Sympathetic activation in rats with L-NAME-induced hypertension. *Braz J Med Biol Res* 2007; **40**: 401–408.
26 Campbell DJ. L-NAME hypertension: trying to fit the pieces together. *J Hypertens* 2006; **24**: 33–36.
27 Zanchi A, Schaad NC, Osterheld MC, Grouzmann E, Nussberger J, Brunner HR, Waeber B. Effects of chronic NO synthase inhibition in rats on renin-angiotensin system and sympathetic nervous system. *Am J Physiol* 1995; **268**: H2267–H2273.
28 Sander M, Hansen PG, Victor RG. Sympathetically mediated hypertension caused by chronic inhibition of nitric oxide. *Hypertension* 1995; **26**: 691–695.
29 Yan W, Sheng ZM, Yu L. Nebivolol treatment improves resistant arterial function and reduces ventricular hypertrophy and angiotensin II in spontaneously hypertension rats. *J Renin Angiotensin Aldosterone Syst* 2013; **14**: 146–155.
30 Varagic J, Ahmad S, Voncannon JL, Moniwa N, Simington SW Jr, Brosnihan BK, Gallagher PE, Habibi J, Sowers JR, Ferrario CM. Nebivolol reduces cardiac angiotensin II, associated oxidative stress and fibrosis but not arterial pressure in salt-loaded spontaneously hypertensive rats. *J Hypertens* 2012; **30**: 1766–1774.
31 Chiladakis JA, Georgiopoulou E, Alexopoulos D. Autonomic effects of nebivolol versus atenolol in healthy subjects. *Cardiovasc Drugs Ther* 2004; **18**: 469–473.
32 Sacco G, Evangelista S, Criscuoli M, Goso C, Bigioni M, Binaschi M, Manzini S, Maggi CA. Involvement of nitric oxide in both central and peripheral haemodynamic effect of D/L-nebivolol and its enantiomers in rats. *Eur J Pharmacol* 2005; **511**: 167–174.
33 Stauss HM. Identification of blood pressure control mechanisms by power spectral analysis. *Clin Exp Pharmacol Physiol* 2007; **34**: 362–388.
34 Janssen BJ, Oosting J, Slaaf DW, Persson PB, Struijker-Boudier HA. Hemodynamic basis of oscillations in systemic arterial pressure in conscious rats. *Am J Physiol* 1995; **269**: H62–H71.
35 Fazan R Jr, Huber DA, Silva CA, Dias da Silva VJ, Salgado MC, Salgado HC. Sildenafil acts on the central nervous system increasing sympathetic activity. *J Appl Physiol* 2008; **104**: 1683–1689.
36 Webb AJ, Fischer U, Mehta Z, Rothwell PM. Effects of antihypertensive-drug class on interindividual variation in blood pressure and risk of stroke: a systematic review and meta-analysis. *Lancet* 2010; **375**: 906–915.
37 Rothwell PM, Howard SC, Dolan E, O'Brien E, Dobson JE, Dahlöf B, Poulter NR, Sever PSASCOT-BPLA and MRC Trial Investigators. Effects of beta-blockers and calcium channel blockers on within-individual variability in blood pressure and risk of stroke. *Lancet Neurol* 2010; **9**: 469–480.
38 Höcht C, Bertera FM, Mayer MA, Taira CA. Issues in drug metabolism of major antihypertensive drugs: beta-blockers, calcium channel antagonists and angiotensin receptor blockers. *Expert Opin Drug Metab Toxicol* 2010; **6**: 199–211.
39 Höcht C, Bertera FM, Taira CA. Importance of blood pressure variability in the assessment of cardiovascular risk and benefits of antihypertensive therapy. *Expert Rev Clin Pharmacol* 2010; **3**: 617–621.
40 Webb AJ, Fischer U, Rothwell PM. Effects of b-blocker selectivity on blood pressure variability and stroke: a systematic review. *Neurology* 2011; **77**: 731–737.
41 Bertera FM, Del Mauro JS, Lovera V, Chiappetta D, Héctor Polizio A, Alberto Taira C, Höcht C. Acute effects of third generation β-blockers on short-term and beat-to-beat blood pressure variability in sinoaortic-denervated rats. *Hypertens Res* 2013; **36**: 349–355.
42 Wang DS, Xie HH, Shen FM, Cai GJ, Su DF. Blood pressure variability, cardiac baroreflex sensitivity and organ damage in experimentally hypertensive rats. *Clin Exp Pharmacol Physiol* 2005; **32**: 545–552.
43 Blanc J, Ponchon P, Laude D, Elghozi JL, Jover B. Blood pressure variability in established L-NAME hypertension in rats. *J Hypertens* 1999; **17**: 1527–1534.
44 Gouédard O, Blanc J, Gaudet E, Ponchon P, Elghozi JL. Contribution of the renin-angiotensin system to short-term blood pressure variability during blockade of nitric oxide synthesis in the rat. *Br J Pharmacol* 1996; **119**: 1085–1092.