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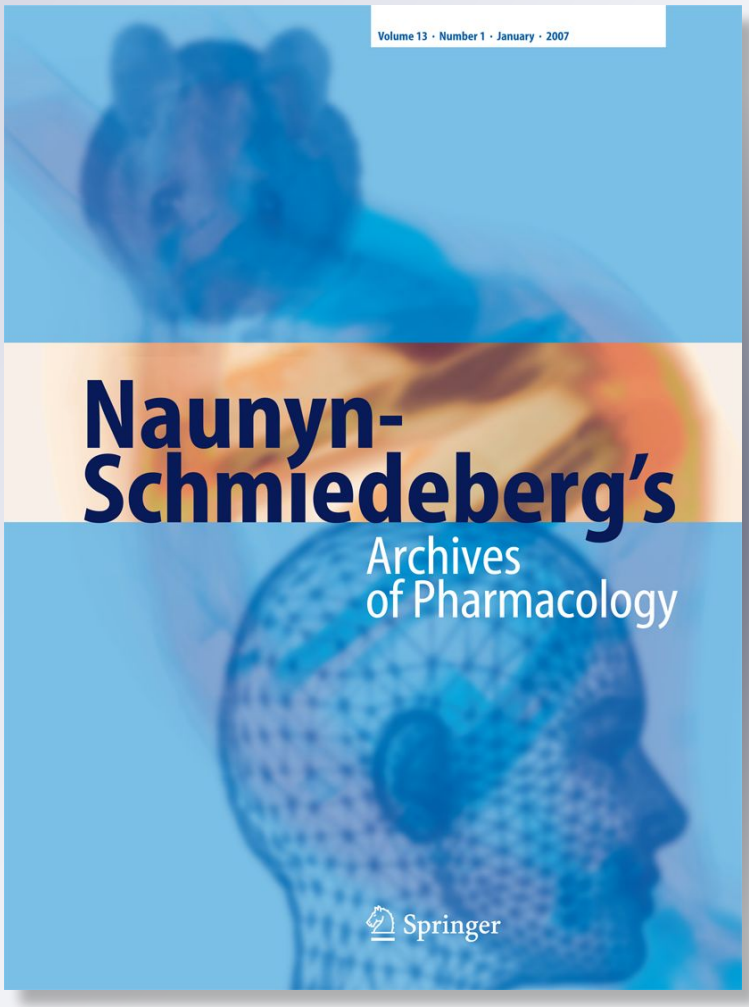

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ORIGINAL ARTICLE

Effect of nebivolol on beat-to-beat and short-term blood pressure variability in spontaneously hypertensive rats

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Abstract Cardiovascular effects and pharmacokinetics of nebivolol were assessed in spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) animals. Male SH and WKY rats were treated with vehicle or nebivolol 0.3, 3, or 10 mg kg^{-1} (i.v.) and effects on blood pressure (BP), heart rate, and blood pressure variability (BPV) were recorded. Plasma pharmacokinetics of *d*- and *l*-nebivolol was studied by traditional blood sampling. Short-term and beat-to-beat BPV was assessed by standard deviation and spectral analysis of BP recording, respectively. Nebivolol showed enantioselective pharmacokinetics in both experimental groups; clearance of *l*-nebivolol was significantly greater than *d*-enantiomer. Clearance of nebivolol was significantly reduced in SHR with regards to WKY animals. Hypotensive response to nebivolol 3 and 10 mg kg^{-1} was significantly enhanced in SHR compared with normotensive animals. Spectral analysis of beat-to-beat BPV showed a greater reduction in low frequency BPV in SHR than in WKY rats. Nebivolol 3 and 10 mg kg^{-1} significantly reduced ratio low frequency/high frequency BPV only in SHR. Short-term BPV was markedly reduced by nebivolol 0.3, 3, and 10 mg kg^{-1} in WKY and SHR. In conclusion, the hypertensive stage in SHR modifies nebivolol pharmacokinetic properties and enhances its hypotensive response due to a greater attenuation in vascular sympathetic activity and enhancement of endothelial-derived NO activity. Nebivolol markedly attenuates short-term BPV in both experimental groups providing beneficial cardiovascular effects by both controlling high blood pressure and its short-term variability.

Keywords Nebivolol · Spontaneously hypertensive rats · Pharmacokinetics · Blood pressure · Sympathetic vascular activity · Blood pressure variability

Introduction

Preclinical and clinical evidences have demonstrated that blood pressure variability (BPV) is an independent risk factor for the incidence of cardiovascular events (Parati 2005; Su and Miao 2001; Rothwell 2011; Mancia and Grassi 2000). Recent data have increased interest in the clinical significance of this cardiovascular parameter, considering the fact that enhanced BPV has been associated with target organ damage in normotensive subjects (Schutte et al. 2011).

Nowadays, it is considered that antihypertensive agents need not only effectively reduce mean arterial pressure but also attenuate its short- and long-term variability (Schillaci et al. 2011). Although β-blockers have been traditionally considered as first-line therapy for hypertension, recently, their role in management of uncomplicated hypertension has been criticized (Tomlinson et al. 2011). In fact, a subanalysis of Anglo-Scandinavian Cardiac Outcomes Trial–Blood

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Pressure Lowering Arm trial results showed that amlodipine exerts greater protection against cerebrovascular events in hypertensive patients than atenolol due to its ability to reduce short- and long-term blood pressure variability (Rothwell et al. 2010). Nevertheless, the extrapolation of these results 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 (Höcht et al. 2010a). Moreover, a recent systematic review has found that variability in systolic blood pressure is increased more by non-selective β-blockers than by selective β_1-adrenergic antagonists (Webb et al. 2011).

Nebivolol belongs to the third generation of β-blockers, which possess direct vasodilator properties in addition to their adrenergic blocking action (Mason et al. 2009). Nebivolol has the highest β_1-receptor affinity among β-blockers, and, most interestingly, it substantially improves endothelial dysfunction by stimulating endothelial nitric oxide synthase activity and via its antioxidative properties.[12] Nebivolol is administered as a racemic mixture (*d*- and *l*-nebivolol) and is extensively metabolized by the cytochrome P-450 2D6 (CYP2D6), with both enantioselective pharmacokinetic and pharmacodynamic properties (Gao and Vanhoutte 2012). Nebivolol enantiomers show differences with respect to their affinity to β-adrenergic receptors. Affinity of *d*-nebivolol by β_1-adrenoceptors is 175-fold higher than the *l*-enantiomer (Gao and Vanhoutte 2012). Nevertheless, *l*-nebivolol potentiates the hypotensive effect of *d*- nebivolol (Gao and Vanhoutte 2012).

The spontaneously hypertensive (SH) rat represents an experimental model of genetic hypertension that develops many features common to human hypertension allowing the study of pathophysiological mechanisms involved in the development and maintenance of essential hypertension and the assessment of pharmacokinetic and pharmacodynamic properties of antihypertensive agents (Pintérová et al. 2011). Although in vivo pharmacodynamic properties of nebivolol have been previously investigated in SHR (Mason et al. 2006; Guerrero et al. 2006; Guerrero et al. 2003; van de Water et al. 1988), these studies show some limitations, including the lack of comparison with normotensive control animals and the absence of the study of pharmacokinetic parameters. In addition, to the best our knowledge, nebivolol effects on blood pressure variability have not been studied in SHR and human subjects.

In accordance, the aim of the present work was the comprehensive assessment of the pharmacokinetics and the in vivo cardiovascular properties of nebivolol, including the effects on heart rate, blood pressure, regulation and its action on beat-to-beat and short-term blood pressure variability in SHR.

Materials and methods

Animals

Male 3-month-old SH (n=24) and WKY rats (n=24) were used (250–270 g). Animal experiments were performed in accordance with the "Principles of laboratory animal care" (NIH publication no. 85-3, revised 1985). Animals were maintained on a 12-h light/dark cycle. Rats were kept in a room at 22±2 °C, and the air was adequately recycled. All animals were fed standard rodent diet (Asociación Cooperativas Argentinas, Buenos Aires, Argentina) with the following composition (*w*/*w*): 20 % proteins, 3 % fat, 2 % fiber, 6 % minerals, and 69 % starch and vitamin supplements, containing the same amount of calories.

Preparation of nebivolol formulation

Nebivolol is practically insoluble in water, and therefore, a special formula was prepared to allow intravenous administration of the drug at a dose of 0.3, 3, and 10 mg kg^{-1}. The formula of nebivolol solution consisted of 0.2 % or 0.5 % (*w*/*v*) nebivolol (gift from Laboratorios Raffo, Buenos Aires, Argentina), 2.0 % (*w*/*v*) polyvinylpyrrolidone, 50 % (*v*/*v*) propylene glycol, 15 % (*v*/*v*) glycerine, and 35 % (*v*/*v*) sorbitol.

Experimental design

Animals were anesthetized with ether, and the left carotid artery and left femoral vein were cannulated with polyethylene cannulae containing heparinized saline solution (25 U ml^{-1}). Cannulae were tunneled under the skin and externalized at the back of the neck. Experiments were performed in freely moving animals 24 h after cannulae placement.

The day of the experiment, arterial cannulae was 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 recordings were stored and analyzed with a software program (Polyview 2.3 Astro-Med, West Warwick, RI, USA). Basal mean arterial pressure (MAP) and heart rate (HR) were estimated during an interval of 60 min previous to 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, at a dose of 0.3, 3, or 10 mg kg^{-1}, or vehicle (n=6 for each group) was injected intravenously during 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. In order to reduce effects attributable to circadian alterations, all experiments were conducted between 1 P.M. and 7 P.M.

Analytical determination of nebivolol

Arterial blood samples (100 μl), collected in polypropylene microcentrifuge tubes containing 5 μl of heparinized solution, were centrifuged at 10,000 rpm for 10 min under controlled temperature (4 °C). It is important to mention that blood sampling could alter 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 concentration 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 (Aimone 2005), and therefore, it could be suggested that blood loss during our experimental protocol did not affect pharmacokinetic and pharmacodynamic properties of nebivolol.

Plasma supernatant (30 μl) was carefully separated, and nebivolol was extracted by 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 30 μl of plasma sample. The mixture was vortexed for 2 min and centrifuged at 2,000 rpm 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.

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, 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/trifluoroacetic acid (65:35:5:0.2). Retention time of *d*-nebivolol and *l*-nebivolol in our chromatographic conditions was 5.7±0.4 and 7.2±0.5 min, respectively. Coefficient of variation of the chromatographic method was <5 %, and limit of quantification of *d*- and *l*-nebivolol was 200 ng ml^{-1}. The intra- and interday coefficients of variation were 4.8 and 4.6, respectively. The method was linear in the range of 200–5,000 ng ml^{-1} and samples with higher concentration of nebivolol were diluted with blank plasma in order to achieve concentrations within the validation range.

Estimation of blood pressure variability

Blood pressure variability (BPV) was continuously estimated by determination of standard deviation (SD) and spectral analysis of 3-min periods of blood pressure recordings obtained from baseline and during regular times after nebivolol administration when the quality of the arterial blood pressure signal was visually considered to be satisfactory. According to previous work by us and other authors,[19,21] spectral analysis of the data was performed using the fast Fourier transform algorithm with a Hamming window (Polyview 2.3 Astro-Med, West Warwick, RI, USA). Spectral densities in the very low frequency range (VLF) (0.1–0.2 Hz), in the low frequency (LF) range (0.2–0.7 Hz), and in the high frequency range (HF) (0.7–2.5 Hz) were calculated from baseline recordings and after nebivolol racemate administration as previously reported (Bertera et al. 2012a,b). Although LF variability is affected by sympathetic modulation of vascular tone, we used 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 (Pladys et al. 2004; Souza et al. 2008).

Pharmacokinetic analysis

Pharmacokinetics of total plasma *d*- and *l*-nebivolol concentrations was estimated by compartmental analysis by applying a two-compartment, first-order elimination model. Nonlinear least squares regression analysis was performed using the TOPFIT program (version 2.0, Dr. Karl Thomae Gmbh, Schering AG, Gödecke AG, Germany) that 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 (AUC) of nebivolol levels vs. time (from 0 to infinity) was calculated using the linear trapezoidal rule. Clearance (Cl) and steady state volume of distribution (Vd_{ss}) were estimated by standard methods (Gibaldi and Perrier 1982).

Statistical analysis

Normal distribution of the data and the variables of the study was verified using the Kolmogorov–Smirnov test. Data were expressed as means±SEM. Basal values of cardiovascular parameters in WKY and SHR were compared by means of Student's *t* test. Statistical analysis of nebivolol effect on MAP, HR, SD, and LF/HF ratio was performed by two-way analysis of variance (ANOVA) and the test of Bonferroni as post hoc test. Pharmacokinetic parameters were log transformed for statistical analysis in order to reduce heterogeneity of the variance and further compared by two-way ANOVA and the test of Bonferroni as post hoc test. Statistical tests were performed using GraphPad Prism

version 5.02 for Windows (GraphPad Software, San Diego, California, CA, USA). Statistical significance was defined as $p<0.05$.

Results

Baseline values of cardiovascular parameters and BPV are shown in Table 1. MAP and short-term BPV were significantly increased in SHR compared with normotensive WKY animals (Table 1). Spectral analysis of blood pressure recording showed an enhanced beat-to-beat BPV in the VLF, LF, and HF domain in hypertensive SHR with regards to WKY animals (Table 1). Vascular sympathetic activity was significantly increased in SHR, considering that LF/HF ratio was greater in hypertensive group (4.56±0.14, $n=24$, $p<0.05$ vs. WKY rats) than in WKY rats (3.71±0.17, $n=24$).

Nebivolol pharmacokinetics

Figure 1 shows the temporal course of *d*-nebivolol and *l*-nebivolol plasma concentrations in control ($n=12$) and SHR ($n=12$) after intravenous 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. Plasma concentrations of both enantiomers of nebivolol were mainly under limit of quantification after administration of 0.3 mg kg^{-1}.

The resulting pharmacokinetic parameters are shown in Table 2. No differences were found in the constant of distribution, Vd_{ss} and C_{max} of both *d*- and *l*-nebivolol in SHR compared with WKY rats. Clearance of *l*-nebivolol was significantly lower in SHR with regards to WKY animals at both dose levels. In addition, a non-significant reduction in clearance of *d*-nebivolol was found in SHR compared with normotensive group. Consequently, terminal half-life of elimination of *l*-nebivolol was reduced in SHR in comparison with WKY animals after application of 10 mg kg^{-1}. Nebivolol showed enantioselective pharmacokinetics considering that clearance of *l*-nebivolol was significantly higher in comparison to *d*-nebivolol in both experimental groups. In addition, AUC of *d*-nebivolol was greater regarding the *l*-enantiomer in SH and WKY rats. Both nebivolol enantiomers showed linear pharmacokinetics; both C_{max} and AUC increased in a dose-dependent manner in both experimental groups.

Table 1 Baseline cardiovascular parameters of Wistar Kyoto (WKY) and spontaneously hypertensive rats (SH) rats

Parameter	WKY rats ($n=24$)	SHR ($n=24$)
Cardiovascular parameters		
MAP (mmHg)	110±4	145±3*
HR (bpm)	349±7	365±7
Blood pressure variability		
Standard deviation (mmHg)	4.48±0.35	6.05±0.39*
VLF variability (mmHg^2)	16.6±1.9	28.9±2.6*
LF variability (mmHg^2)	12.0±1.4	25.0±2.0*
HF variability (mmHg^2)	3.3±0.5	5.9±0.6*

*$p<0.05$ vs. WKY rats by Student's *t* test

Effects of nebivolol i.v. administration on heart rate

Figure 2 shows the temporal course of HR changes in WKY and SHR after vehicle or nebivolol intravenous administration at a dose of 0.3, 3, or 10 mg kg^{-1}. Vehicle administration did not modify HR in either experimental group. Nebivolol induced a dose-dependent reduction of HR in both experimental groups (Fig. 2) with partial recovery of baseline HR values during follow-up. Chronotropic response to nebivolol did not differ between SH and WKY rats (Fig. 2) comparing three dose levels.

Effects of nebivolol i.v. administration on blood pressure

Hypotensive response to i.v. administration of nebivolol at a dose of 0.3, 3. or 10 mg kg^{-1} is depicted in Fig. 3. No changes in MAP were found in SH and WKY rats after vehicle administration. Nebivolol 0.3 mg kg^{-1} induced a slight but significant reduction in blood pressure of both WKY and SHR, without differences between experimental groups (Fig. 3). Increase in nebivolol dose to 3 or 10 mg kg^{-1} induced a marked decrease in MAP in WKY and SHR. Moreover, at these dose levels, hypotensive response to nebivolol was significantly enhanced in hypertensive SH animals compared with normotensive WKY rats (Fig. 3). MAP did not return to baseline values during the entire experiment in both experimental groups demonstrating the long lasting antihypertensive response to nebivolol i.v. application (Fig. 3).

Effects of nebivolol i.v. administration on beat-to-beat and short-term blood pressure variability

Nebivolol i.v. administration dramatically reduced short-term BPV in both normotensive WKY and hypertensive SHR as evidenced by the sustained reduction in SD of blood pressure after administration of 0.3, 3. or 10 mg kg^{-1}of the drug (Fig. 4). No differences were found in the magnitude of SD attenuation comparing SH and WKY groups. Moreover, increase in nebivolol dose from 0.3 mg kg^{-1} to 3 or 10 mg kg^{-1} did not further reduce SD of blood pressure recording in WKY and SHR, suggesting the absence of a dose–response relationship for nebivolol effects on short-term BPV.

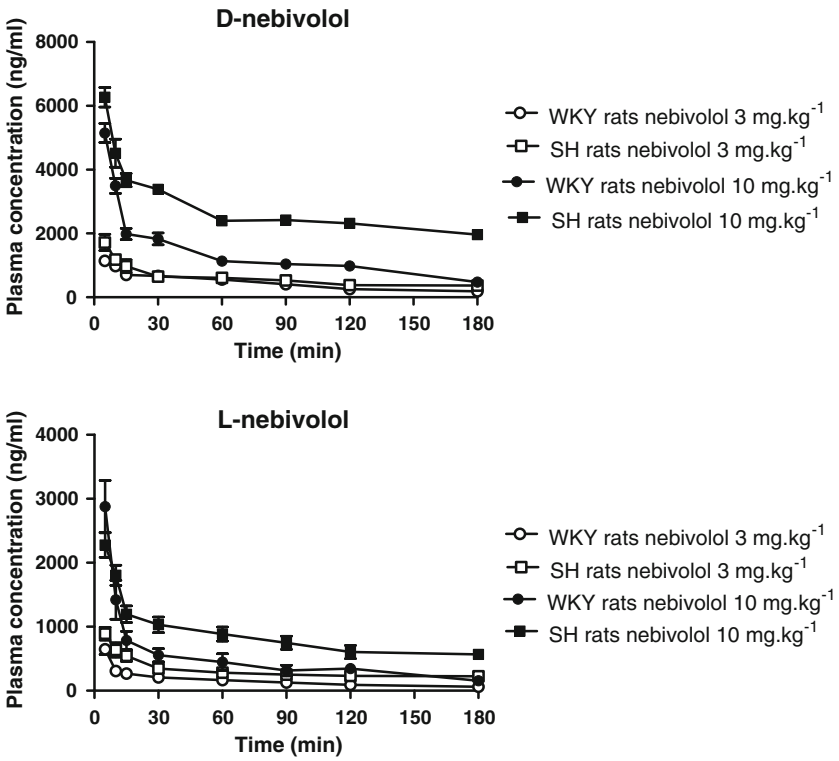

Fig. 1 Mean plasma concentration values of *d*-nebivolol and *l*-nebivolol vs. time in normotensive WKY rats (*circles*) and SH animals (*squares*) after administration of 3 mg kg^{-1} (*open symbols*) and 10 mg kg^{-1} (*black symbols*) of the drug. Each point shows the mean±SEM of six rats

Beat-to-beat BPV was also significantly reduced by nebivolol administration in both experimental groups. Neither vehicle nor nebivolol 0.3 mg kg^{-1} modify BPV at the three domains (VLF, LF, and HF; Fig 5). In WKY rats, nebivolol 3 and 10 mg kg^{-1} induced a significant reduction of VLF and LF BPV. Conversely, all three domains of beat-to-beat BPV were attenuated by nebivolol 3 and 10 mg kg^{-1} in SH animals. Moreover, nebivolol exerted a greater decrease in LF variability in SHR compared with WKY animals at both dose levels (Fig. 5). Effect of nebivolol on VLF and HF BPV did not differ between both experimental groups (Fig. 5).

Finally, nebivolol effects on LF/HF ratio, a marker of vascular sympathetic activity, were also assessed in WKY and SHR after administration of vehicle or nebivolol 0.3, 3, or 10 mg kg^{-1}. While nebivolol 0.3 mg kg^{-1} did not modify the LF/HF ratio in both experimental groups, the higher dose levels induced a significant reduction of this parameter in SHR but not in WKY animals (Fig. 6).

Discussion

The present study was designed to compare pharmacokinetic and cardiovascular properties of nebivolol in SH and WKY rats with special focus in drug effects on BPV. Cardiovascular effects and the mechanism of action of nebivolol have been extensively studied in hypertensive animals. Nebivolol shows enantioselective pharmacodynamic properties due to the highly selective β_1-adrenoceptor blocking activity of *d*-nebivolol and direct vasodilatory action of both enantiomers. Mechanisms involved in reduction of vascular tone induced by nebivolol include stimulation of endothelial-dependent NO synthase and inhibition of endothelial dysfunction through a potent antioxidant mechanism attributed to nebivolol interaction with the membrane (Gao and Vanhoutte 2012; Mason et al. 2006). Previous studies in SHR have demonstrated that nebivolol produces sustained reductions in blood pressure and heart rate after acute and chronic administration (Guerrero et al. 2006; van de Water et al. 1988). Nevertheless, to the best our knowledge, pharmacokinetic and cardiovascular properties of nebivolol in SHR were not previously compared with normotensive WKY animals.

Nebivolol pharmacokinetics was studied 24 h after arterial cannulation. It has been demonstrated that surgical implantation of cannulae 24 h before the experiment induced an increment of α_1-glycoprotein (Terao and Shen 1983). As nebivolol binds predominantly to serum albumin (Gao and Vanhoutte 2012), it seems unlikely that an increase in α_1-glycoprotein due to cannulae implantation would affect drug pharmacokinetics in our experimental conditions. Nebivolol pharmacokinetics has been studied previously in human subjects. This β-blocker undergoes high hepatic extraction and is extensively metabolized by the cytochrome P450 2D6 to its hydroxilated active metabolite (Gao and Vanhoutte 2012). In addition, nebivolol exhibits stereoselective pharmacokinetic properties, considering that peak and trough plasma concentrations of *d*-nebivolol were

Table 2 Pharmacokinetic parameters of total *d*-nebivolol and *l*-nebivolol plasma levels obtained from arterial blood samples: area under the curve (AUC), α (constant of distribution), β (constant of elimination), Cl (clearance) and Vd_{ss} (steady state volume of distribution), C_{max} (extrapolated maximal concentration) in WKY and SHR after i.v. administration of drug (3 and 10 mg kg^{-1})

Enantiomer	*d*-Nebivolol				*l*-Nebivolol			
Experimental group	WKY rats (n=12)		SHR (n=12)		WKY rats (n=12)		SHR (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})	7.2±2.9	8.3±2.0	7.5±1.3	11.2±2.1	10.1±0.7	10.7±1.7	7.5±1.5	6.7±0.7
β (h^{-1})	0.39±0.05	0.29±0.07	0.24±0.05	0.41 ±0.04	0.45±0.03	0.47±0.13	0.38±0.08	0.23±0.05***
Vd_{ss} (l)	2.3±0.5	4.0±1.2	2.4±0.4	1.5±0.3	5.6±1.6**	4.7±0.6	3.5±0.4	5.4±1.1**
Cl (ml min^{-1})	18.8±5.0	19.4±6.1	10.6±3.5	13.4±2.7	59.9±16.9**	47.8±9.5	22.4±6.2***	19.3±3.5***
C_{max} (ng ml^{-1})	1,777±216	6,665±1,352*	2,233±553	7,770±1,216*	1,270±215	5,060±773*	1,318±204	2,507±216***
$AUC_{0\to\infty}$ (ng ml h^{-1})	1,742±452	5,755±1,670*	3,480±834	7,244±1,298*	582±217**	2,110±629*	1,666±340***	5,008±984*****
r^2	0.995 (0.992–0.998)	0.997 (0.994–0.999)	0.996 (0.985–0.999)	0.997 (0.995–0.999)	0.996 (0.993–0.999)	0.997 (0.994–0.999)	0.997 (0.994–0.999)	0.992 (0.967–0.999)
AIC	81.7 (57.6–92.8)	81.6 (65.6–118)	78.3 (66.2–89.4)	89.8 (64.2–112)	64.1 (57.0–75.0)	73.9 (55.4–91.6)	46.7 (14.9–65.2)	68.5 (31.6–98.2)

Data are expressed as mean±SEM. Goodness of fit indicators are expressed as mean (range)

*p<0.05 vs. 3 mg kg^{-1} by two-way ANOVA followed by Bonferroni post-test; **p<0.05 vs. *d*-nebivolol by two-way ANOVA followed by Bonferroni post-test; ***p<0.05 vs. WKY rats by two-way ANOVA followed by Bonferroni post-test

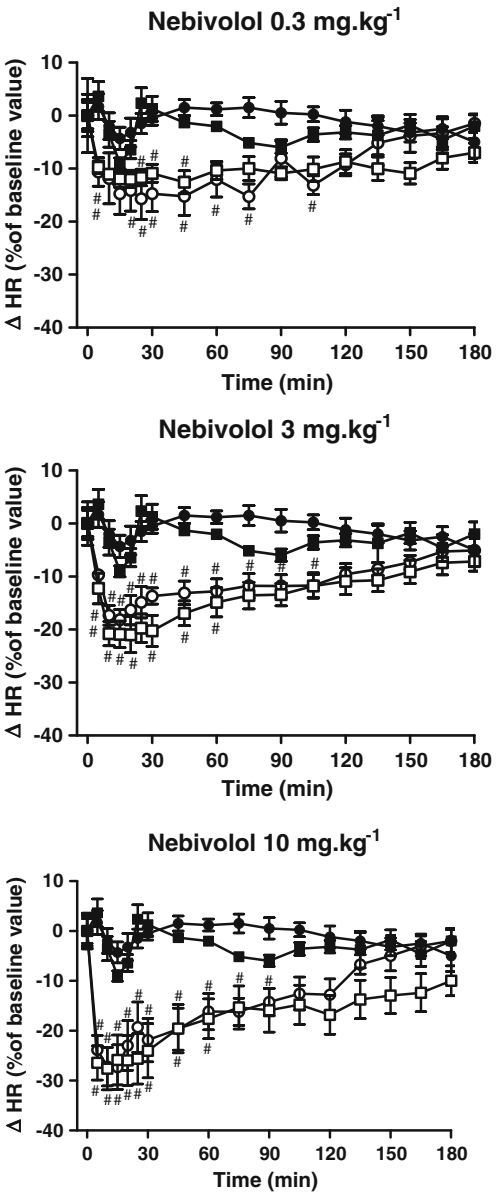

Fig. 2 Time course of changes in heart rate (ΔHR, percent of baseline values), after i.v. administration of nebivolol (*open symbols*) or vehicle (*black symbols*) in normotensive WKY rats (*circles*) and SH animals (*squares*). Each point shows the mean±SEM of six rats. #p<0.05 vs. vehicle by two-way ANOVA followed by Bonferroni post-test

greater than the *l*-enantiomer after single and multiple administrations (Höcht et al. 2010b). The present study confirms enantioselective pharmacokinetic pattern of nebivolol in both normotensive and hypertensive animals. Clearance of *l*-nebivolol was significantly greater than *d*-nebivolol resulting in reduced AUC in WKY and SHR.

Comparison of pharmacokinetic parameters between both experimental groups showed that the hypertensive stage in SHR affects drug clearance of *d*- and *l*-nebivolol compared with normotensive WKY animals. Although the mechanisms involved in this finding are unclear, considering the fact that

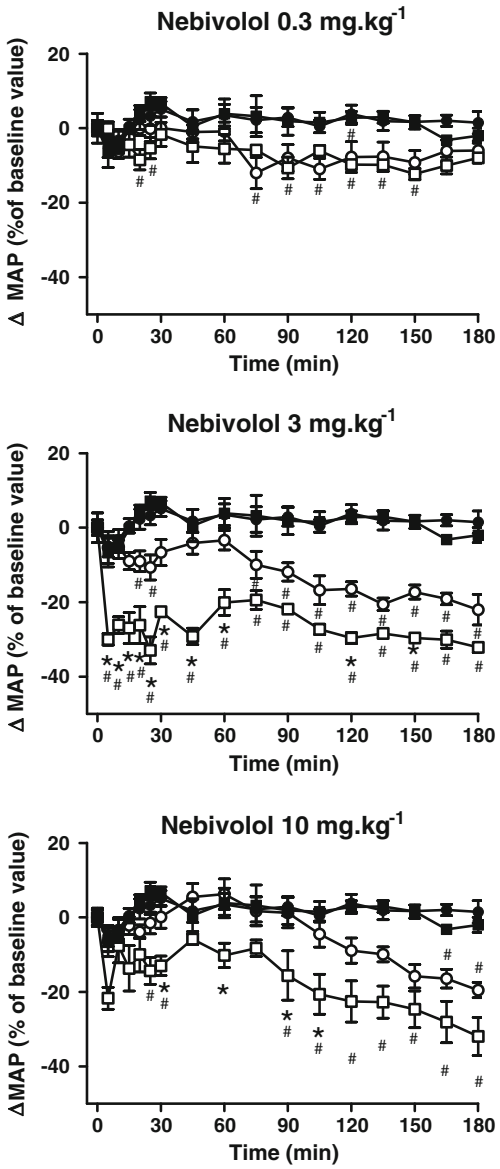

Fig. 3 Time course of changes in mean arterial pressure (ΔMAP, percent of baseline values), after i.v. administration of nebivolol (*open symbols*) or vehicle (*black symbols*) in normotensive WKY rats (*circles*) and SH animals (*squares*). Each point shows the mean±SEM of six rats. $^{\#}p<0.05$ vs. vehicle, $^{*}p<0.05$ vs. WKY rats by two-way ANOVA followed by Bonferroni post-test

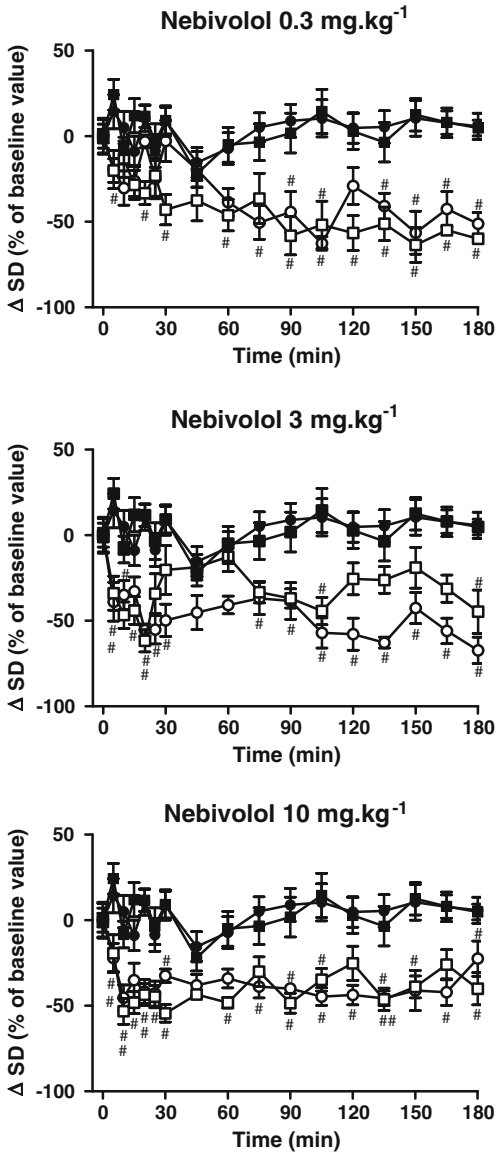

Fig. 4 Change in short-term blood pressure variability, expressed as percent of baseline standard deviation (SD) value, after i.v. administration of nebivolol (*open symbols*) or vehicle (*black symbols*) in normotensive WKY rats (*circles*) and SH animals (*squares*). Each point shows the mean±SEM of six rats. $^{\#}p<0.05$ vs. vehicle by two-way ANOVA followed by Bonferroni post-test

nebivolol clearance depends on hepatic blood flow (Höcht et al. 2010b), our results suggest a lower hepatic perfusion in SHR compared with WKY animals after administration of nebivolol 3 and 10 mg kg^{-1}. Supporting this finding, Isanta et al. (1987) have also described a reduced plasma clearance of diltiazem, a calcium channel blocker with high hepatic extraction (Höcht et al. 2010b), in SHR with regards to normotensive WKY animals.

Nebivolol i.v. administration induced a dose-dependent reduction in blood pressure in both experimental groups, considering that nebivolol 0.3 mg kg^{-1} only slightly decrease MAP in both WKY and SH animals in contrast to the marked blood pressure reduction after application of higher dose levels. Analysis of the temporal course of blood pressure response to nebivolol administration showed in SHR both an early and late reduction in MAP. In contrast, early hypotensive response to nebivolol was mainly absent in WKY animals. Considering the fact that chronotropic response to nebivolol was similar comparing both experimental groups, it could be suggested that increase in early blood pressure lowering effect of nebivolol in SHR is mainly due to enhanced reduction in systemic resistance in hypertensive animals. The lower

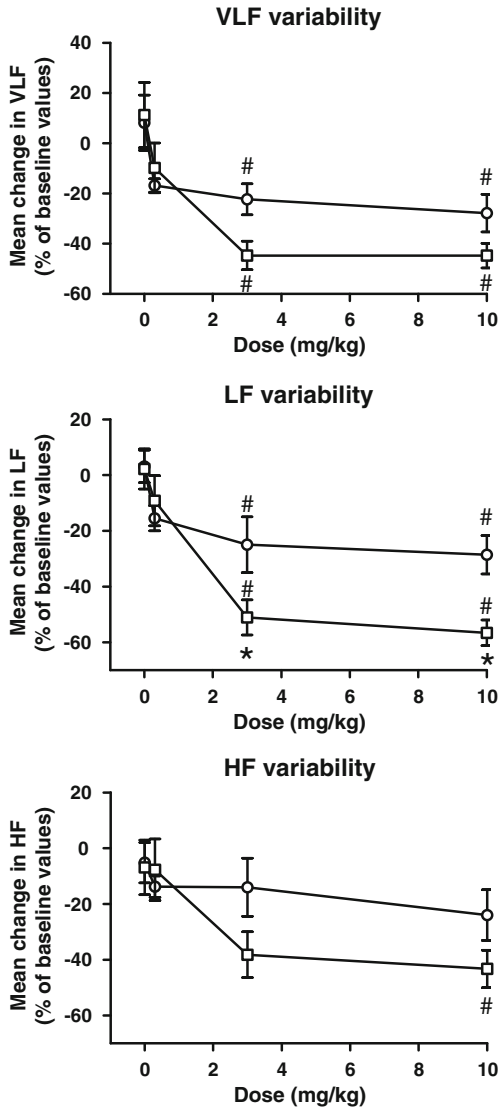

Fig. 5 Dose–response of blood pressure variability in the very low frequency (VLF), low frequency (LF), and high frequency (HF) domain (expressed as percentage of baseline values) in WKY rats (*circles*) and SH animals (*squares*) after administration of vehicle or iv nebivolol. $^{\#}p<0.05$ vs. vehicle, $*p<0.05$ vs. WKY rats by two-way ANOVA followed by Bonferroni post-test

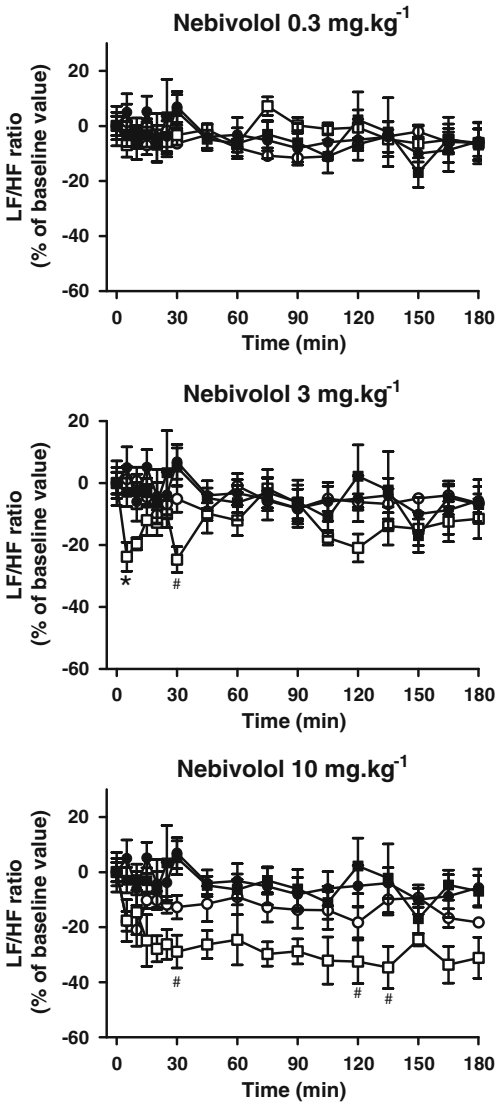

Fig. 6 Time course of changes in normalized low frequency (LF) variability (LF/HF ratio), expressed as % of baseline values, after iv administration of nebivolol (*open symbols*) or vehicle (*black symbols*) in normotensive WKY rats (*circles*) and SH animals (*squares*). Each point shows the mean±SEM of six rats. $^{\#}p<0.05$ vs. vehicle, $*p<0.05$ vs. WKY rats by two-way ANOVA followed by Bonferroni post-test

contribution of reduction in cardiac output in the early hypotensive response to nebivolol is also sustained by the fact that the lower dose of nebivolol (0.3 mg kg^{-1}) induced a significant reduction in HR during the first 30 min with only slightly effects on MAP. An interesting finding of the hypotensive response to nebivolol in SHR is the observation of a lower early hypotensive action after administration of the highest dose level with regard to nebivolol 3 mg kg^{-1}. Paradoxical attenuation of early hypotensive response by dose increment of nebivolol could be explained by results by Van de Water et al. (1988). These authors have found a lower reduction of systemic vascular resistance after application of higher doses of nebivolol with respect to low doses (Van de Water et al. 1988).

In addition, nebivolol exerted a sustained hypotensive response in both experimental groups, taking into account that blood pressure did not return to baseline values during the entire experiment. Sustained hypotensive response to nebivolol has been previously described by other authors (van de Water et al. 1988). In agreement with our results, van de Water et al. (1988) have found that blood pressure reduction after intraperitoneal administration of nebivolol at a dose of 1.25 mg kg^{-1} in awake SHR was maximal after 180 min of drug administration. This pharmacodynamic pattern of nebivolol greatly contrasts with other β-blockers, such as metoprolol and carvedilol. In previous studies, we have found a fast recovery of MAP in WKY and SHR after

administration of carvedilol or metoprolol (Bertera et al. 2012b; Höcht et al. 2006). Several mechanisms could contribute to the sustained hypotensive efficacy of nebivolol, including its slow dissociation rate from the receptor and the generation of active metabolites (Kuroedov et al. 2004). Meier et al. (2004) have found a long-lasting hypotensive response to acute nebivolol administration in SHR that has been attributed to its active metabolites.

Comparison of nebivolol effects on blood pressure in WKY and SHR demonstrated the enhanced hypotensive response to beta blocker in hypertensive rats. Although increased blood pressure lowering response to nebivolol in SH animals could be attributed to higher drug levels when compared with WKY rats, it seems unlikely that greater drug exposure plays a role in the enhancement of nebivolol actions in the hypertensive group. In fact, clinical studies have shown that antihypertensive response to nebivolol is not related to plasma concentration of the β-blocker (Lefebvre et al. 2007). Even though poor metabolizer patients show steady-state plasma concentrations of *d*- and *l*-nebivolol 10- and 15-fold greater than extensive metabolizers, chronic administration of nebivolol produced similar efficacy and tolerability in hypertensive patients either characterized as poor or extensive metabolizers (Lefebvre et al. 2007).

Spectral analysis of the blood pressure recording suggests that the greater hypotensive response of nebivolol in SHR could be explained by the increased ability of the drug to enhance NO-dependent vasodilation and to reduce vascular sympathetic activity. Identification of the frequency components of blood pressure variability by power spectral analysis can potentially provide information about mechanisms involved in blood pressure regulation (Stauss 2007). In this context, while myogenic vascular function affects blood pressure variability at VLF (Langager et al. 2007), LF variability is modulated by sympathetic modulation of vascular tone and endothelial-derived NO in rats (Stauss 2007). In addition, normalized LF (LF/HF ratio) has been validated as a marker of sympathetic vascular activity in preclinical and clinical studies (Souza et al. 2008; Fazan et al. 2008). Variability in the HF domain is mainly influenced by changes in cardiac output (Janssen et al. 1995). Although nebivolol administration induced a marked reduction of beat-to-beat BPV in both experimental groups, decrease in LF variability was greater in SHR compared with WKY animals, suggesting that enhancement of endothelial-derived NO partially explains greater hypotensive response to nebivolol in hypertensive animals. In addition, administration of hypotensive doses of nebivolol (3 or 10 mg kg^{-1}) reduced the LF/HF ratio in SHR but not in normotensive WKY animals, suggesting that attenuation of vascular sympathetic activity could also contribute to the enhanced hypotensive response to nebivolol in SHR. Several previous works have described sympatholytic actions of nebivolol, which are partially related to the increase in NO bioavailability (Chiladakis et al. 2004; Sacco et al. 2005). Sacco et al. (2005) have found that central application of nebivolol acutely increase brain NO release reducing thereby activity of the sympathetic nervous system.

The main objective of the present work was the assessment of the acute effects of nebivolol administration in short-term BPV in WKY and SH animals estimated by the SD of the blood pressure recording. Recent findings have highlighted the role of BPV on cardiovascular morbidity and mortality in both normotensive and hypertensive subjects (Rothwell 2011). Excessive fluctuation of blood pressure plays important roles in the progression of organ damage and in triggering vascular events, particularly stroke (Rothwell 2011). Moreover, increased BPV has been associated with left ventricular hypertrophy in normotensive subjects (Schutte et al. 2011). Considering the contribution of BPV in the development of cardiovascular events, reduction of this parameter should be considered as a possible new target in cardiovascular medicine (Schillaci et al. 2011).

Clinical trials have demonstrated that β-blockers, such as atenolol, are not able to reduce and rather increase BPV in hypertensive subjects (Webb et al. 2011). However, the extrapolation of these results to third generation β-blockers, such as nebivolol, seems not to be adequate considering the fact that β-blockers greatly differ in their pharmacokinetic and pharmacodynamic properties (Cockcroft and Pedersen 2012). Effects of β-blockers on BPV in SHR have been previously reported by other authors with conflicting results. Long-term treatment with atenolol markedly reduced blood pressure variability, enhanced baroreflex sensitivity, and produced significant organ protection in SHR (Xie et al. 2006). Conversely, acute administration of metoprolol did not affect BPV of MAP during both light and dark hours and circadian variability of blood pressure (Janssen et al. 1991; Friberg et al. 1998). To the best our knowledge, nebivolol effects on BPV have not been previously studied in normotensive and hypertensive subjects.

Compared with WKY animals, SHR showed higher baseline SD values confirming that short-term BPV increases with blood pressure levels. In agreement with our findings, Miao et al. (2006) have previously found an increase in systolic and diastolic short-term BPV in SHR with regards to normotensive WKY animals. Nebivolol i.v. administration dramatically reduced SD of blood pressure recording in both experimental groups, suggesting that this third generation β-blocker does not only effectively control the hypertensive stage but also attenuates short-term BPV. Moreover, reduction in short-term BPV induced by nebivolol is not related to its blood pressure lowering action; BPV was dramatically reduced in both SHR and WKY rats even by low nebivolol dose (0.3 mg/kg), which exerts minimal blood

pressure lowering effects. In addition, while no dose–response relationship was found for nebivolol effect on short-term BPV in WKY and SHR, reduction of MAP was significantly greater after administration of 3 and 10 mg kg^{-1} compared with the lower dose. Furthermore, although nebivolol showed higher hypotensive efficacy in SHR than in WKY animals, attenuation of short-term variability was similar between both experimental groups. Therefore, our results suggest that BPV reduction is not associated to nebivolol effect on average blood pressure.

Long-term studies are needed to clarify if nebivolol effect on BPV is able to reduce target organ damage in SHR even at non-depressor doses. Clinical and experimental evidences suggest that non-depressor doses of antihypertensive drugs are capable to reduce target organ damage through attenuation of BPV. Schutte et al. (2011) have shown that increased BPV is associated with left ventricular hypertrophy in normotensive subjects. In addition, Xie et al. (2005) have demonstrated that treatment of SHR with low-dose ketanserin reduces BPV and protects from target organ damage without significantly reduction of blood pressure.

The findings of the present study provide new insights in the pharmacokinetic and cardiovascular properties of the third generation β-blocker nebivolol in experimental models of hypertension. Enantioselective pharmacokinetics of nebivolol was found in WKY and SHR considering the fact of increased systemic clearance of *l*-nebivolol with regards to the *d*-enantiomer. Nebivolol pharmacokinetics was affected by the hypertensive stage in SH animals with a reduced drug clearance when compared with normotensive WKY rats. Acute administration of nebivolol exerts a sustained and dose-dependent hypotensive response in both WKY and SHR. Moreover, blood pressure lowering efficacy is significantly enhanced in SHR compared with normotensive WKY rats probably by enhancement of endothelial-derived NO activity and reduction of vascular sympathetic activity. Nebivolol markedly attenuates 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 blood pressure and its short-term variability. Interestingly, short-term BPV reduction induced by nebivolol is evident at low dose levels associated with limited effects on blood pressure. Additional studies are needed to establish if the ability of nebivolol to attenuate short-term BPV translates into a greater target organ damage protection in hypertensive subjects.

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