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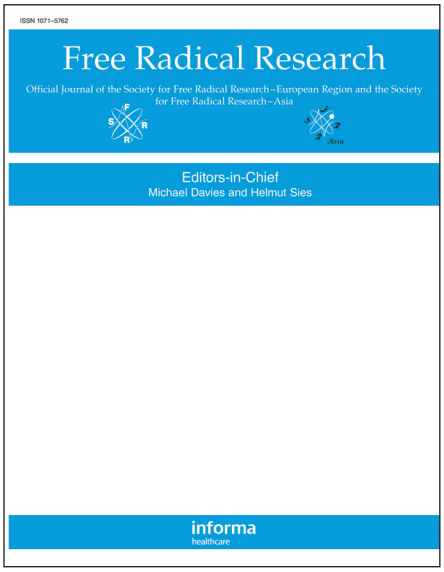

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Facundo M. Bertera, Diego M. Santa-Cruz, Karina B. Balestrasse, Susana B. Gorzalczany, Christian Höcht, Carlos A. Taira, Ariel H. Polizio

10.3109/10715762.2013.845294

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Male Sprague Dawley rats were given tap water to drink (control group) or tempol (an antioxidant scavenger of superoxide) for 1 week. After 1 week, Nebivolol, at a dose of 3 mg/kg was injected intravenously to the control group or tempol treated group. Mean arterial pressure, heart rate and blood pressure variability were evaluated in the control, tempol, nebivolol and tempol nebivolol groups, as well as, the effect of different inhibitor as N^W-nitro-l-arginine methyl ester (L-NAME, a Nitric oxide synthase blocker) or glybenclamide, a K_{ATP} channel inhibitor. Also, the expression of α,β soluble guanylate cyclase (sGC), phospho-eNOS, and phospho-vasodilator-stimulated phosphoprotein (P-VASP) were evaluated by Western Blot and cyclic guanosine monophosphate (cGMP) levels by an enzyme-linked immunosorbent assay (ELISA) commercial kit assay.

We showed that preteatment with tempol in normotensive rats produces a hypotensive response after nebivolol administration through an increase in the NO bioavailability and sGC, improving the NO/ cGMP/ protein kinase G (PKG) pathway compared to the nebivolol group.

We demonstrated that tempol preadministration beneficiates the response of a third generation beta blocker with eNOS stimulation properties, decreasing the basal uncoupling of eNOS and improving NO bioavailability. Our results clearly open a possible new strategy therapeutic for treating hypertension.

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Short title: Antioxidant pretreatment improves nebivolol action

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Keywords: antioxidants, cardiovascular therapeutic, endothelial nitric oxide, nitric oxide, oxidative stress, third generation beta blockers.

Abbreviations

cyclic guanosine monophosphate , cGMP; endothelial nitric oxide synthase; enzyme-linked immunosorbent assay , ELISA; eNOS; guanosine-5'-triphosphate , GTP; heart rate, HR; hydrogen peroxide , H_2O_2 ; low frequency range , LF; mean arterial pressure, MAP; NW-nitro-l-arginine methyl ester, L-NAME; nitric oxide, NO; NO metabolites content, NOx; peroxynitrite , $ONOO^-$; phospho- vasodilator-stimulated phosphoprotein , P-VASP; protein kinase G, PKG; reactive oxygen species, ROS; soluble guanylate cyclase, sGC; superoxide, $O_2^{\cdot-}$; thiobarbituric acid reactive substances, TBARS; very low frequency range, VLF.

1. Introduction

Oxidative stress is an imbalance between the production and degradation of reactive oxygen species (ROS), such as $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2), thereby leading to cellular and tissue injury due to the high reactivity properties of these oxygen species [1]. The damage inflicted by ROS, as well as, reactive nitrogen species such as peroxynitrite ($ONOO^-$) on cellular and extracellular targets as membrane lipids [2], proteins [3], and deoxyribonucleic acid [4] contributes to tissue and organ dysfunction in several pathologies such as hypertension among others [5]. $O_2^{\cdot-}$ and H_2O_2 produced mainly by the NADPH oxidases in vascular cells are two of the most biologically important ROS in the cardiovascular system [6-8]. Importantly, NAD(P)H production of $O_2^{\cdot-}$ is constitutive conversely to the phagocytic isoform that is only activated when the respiratory burst [5]. Constitutive production leads to basal level of $O_2^{\cdot-}$ also in normotensive animals [9]. In the vasculature, $O_2^{\cdot-}$ reacts with NO from the eNOS to form $ONOO^-$, a potent oxidant compound, leading to uncouple the eNOS [10]. Uncoupling of eNOS generates $O_2^{\cdot-}$ instead of NO. As a consequence, the loss of bioavailable NO and formation of $ONOO^-$ lead to vascular inflammation and remodeling, altered vascular tone, enhanced vascular permeability, and increased platelet aggregation not only in hypertensive patients, but also in normotensive subjects, Consequently, eNOS uncoupling and reduced endothelium-dependent relaxation impair vascular function that leading to hypertension [7,10].

The third generation beta blocker nebivolol represents an attractive option for the treatment of different cardiovascular diseases, including hypertension and cardiac failure. Beneficial properties of nebivolol include the highly selective blockade of the β_1-adrenoceptor and enhancement of NO dependent vasodilation [11-12].

Tempol is an antioxidant that mimics the action of the superoxide dismutase enzyme, that catalyses the conversion of $O_2^{\cdot-}$ into H_2O_2, acting as an efficient scavenger of free radicals [13]. Although the protective effects of tempol against free radical-induced damage have mainly been attributed to the dismutation of $O_2^{\cdot-}$, tempol is a multifunctional antioxidant since it reacts with a diversity of biological oxidants and reductants [14,15].

Munzel et al. have recently reported that those patients with evidence of vascular oxidative stress have a worse prognosis in the management of hypertension [16]. It stands to reason that antioxidants should be beneficial in preventing and/or delaying hypertension. Moreover, it has been demonstrated that chronic treatment with acetylsalicylic acid lowers basal levels of $O_2^{\cdot-}$ in normotensive rats. However, pitfalls of conventional antioxidant therapy in conjunction with the lack of proven benefit of antioxidants have led to suggest that the use of supplemental antioxidant could even be hazardous to patients resulting in an “antioxidant paradox” [17]. Nevertheless, it is important to note that basic and clinical evidences showing cardiovascular benefits of antioxidant supplement are lacking.

Taking into account that the mechanism of action of nebivolol includes eNOS stimulation, we hypothesized that administration of tempol, a $O_2^{\cdot-}$ scavenger, could decrease basal levels of ROS produced by the constitutive

action of vascular NAD(P)H oxidase improving the hypotensive response of nebivolol via NO increased bioavailability.

2. Methods

2.1 Chemicals

Glybenclamide, N-acetyl-cysteine and thiobarbituric acid were from Sigma (St.Louis,MO,USA). Metoprolol and atenolol were purchased from Droguerias Saporiti (Buenos Aires, Argentina). Nebivolol was a gift from Raffo Laboratory (Buenos Aires, Argentina). All other reagents were of analytical grade or better.

2.2 Animals

2.2.1 Animals and chronic treatment of tempol

Male Sprague Dawley 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 Principles of Laboratory

Animal Care (NIH publication 2002 and 2011). The animal experiments 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. Rats were randomly divided into two groups. Control rats (C, n = 46) were given tap water to drink and tempol group (T, n = 60) was given tempol solution at a concentration of 172 mg/L to drink for 1 week [14]. For the N-acetyl-cysteine group (n=5), the drug was given in the drinking water at 4 g/kg/day during one week.

2.3 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 3 mg/kg. The formula of nebivolol solution consisted of 2 mg/ml nebivolol, 0.5%(w/v) polyvinylpyrrolidone, 40% (v/v) propylene glycol, 10% (v/v) glycerine and purified water.

2.4 In vivo experiments

2.4.1 In-vivo experimental design

Rats were anaesthetized with ether and the left carotid artery and left femoral vein were cannulated with polyethylene cannulae containing heparinized saline solution (25 U/ml) for blood pressure recording and drug administration, respectively. Cannulae were tunnelled under the skin and externalized at the back of the neck. Experiments were performed in freely moving animals 24 h after cannulae placement. On the day of the experiment, arterial cannulae were connected to a Spectramed P23XL pressure transducer (Spectramed, Oxnard, CA, USA) coupled to a Grass 79D polygraph (Grass Instruments, Quincy, MA, USA). The polygraph was connected to a digital converter adaptor unit (Polyview, PVA 1; Grass-AstroMed,WestWarwick, RI,

USA), and recordings were stored and analysed with a software program (Polyview 2.3;Astro-Med,WestWarwick, RI, USA). Basal mean arterial pressure (MAP) and heart rate (HR) were estimated during an interval of 45 minutes. 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 3 mg/kg was injected intravenously during 30 s to the control group (CN, n=7) or tempol treated group (TN, n=7). To test different β-blockers responses, metoprolol (3 mg/kg, i.v., n=5) or atenolol (3 mg/kg, i.v., n=5) was given to the T group. To determine the role of NOS or K_{ATP} channels in the cardiovascular effects of nebivolol, the blood pressure response was evaluated in rats pretreated, 45 min before nebivolol administration, with L -NAME (75 mg/kg, i.v.) or glybenclamide (20mg/kg) in the TN group (n = 5, in each group). The indicated dose of L-NAME was chosen because it is specific only for the eNOS isoform [18].

2.4.2 Spectral analysis

Blood pressure variability was continuously estimated by determination of standard deviation (SD) and spectral analysis of 3 min periods of blood pressure recordings were 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 other authors [19,20], spectral analysis of the data was performed using the Fast Fourier Transform algorithm with a Hamming window (Polyview 2.3;Astro-Med). Spectral densities in the very low frequency range (VLF) (0.1–0.2 Hz) and in the low frequency range (LF) (0.2 to 0.7 Hz) were estimated [20].

2.5 Ex vivo experiments

2.5.1 Lipid peroxidation in control and tempol groups

The thoracic aortic rings from the two groups were excised (C and T), washed with ice-cold saline solution (0.9% wt/vol NaCl), and weighed. Homogenate lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS) (expressed as malondialdehyde equivalents) in the C and T, 7 animals in each group as we previously described [21].

2.5.2 Expression of α, β sGC isoforms, phospho-eNOS, eNOS and P-VASP by Western Blot

The animals were sacrificed by cervical dislocation, and thoracic aortic segments from the four experimental groups (C, T, CN and TN) were excised, washed with ice-cold saline solution (9 g/l NaCl) and weighed. Aortic homogenates from C, T for alpha (α), beta (β) guanylate cyclase and C,T, CN and TN groups for phospho-eNOS (Ser1177) and P-VASP (Ser 239) were prepared in a Potter-Elvehjem homogenizer using radioimmuno precipitation assay and Roche Complete Protease inhibitor tablets. The homogenates were then separated by centrifugation at 6,000 g for 20 min, and the supernatant was mixed with Laemmli 6 X loading buffer and incubated at 92 ° C for 5 min. Total tissue protein was separated by electrophoresis in 4-20 % Tris-glycine SDS polyacrylamide gels (Mini Protean III System; BioRad Laboratories, Hercules,

Calif., USA), transferred onto nirocellulose membranes and blocked in 50 g/l dry milk or bovine serum albumin in T-TBS (0.02 mol/l Tris, 0.15 mol/l NaCl, pH 7.5, containing 1 g/l Tween 20) at room temperature for 1 h. The membranes were washed 3 times with T-TBS and incubated with the primary antibodies against alpha (α), beta (β) sGC, phospo-eNOS (Ser 1177), total eNOS and P-VASP (Ser 239) overnight at 4° C. The polyclonal antibodies against α, β guanylate cyclase, were purchased from Sigma (St. Louis, MO., USA), and the monoclonal antibodies against phospo-eNOS, total eNOS and P-VASP were purchased from Cell Signaling Technology, Inc. (Danvers, Mass., USA). After washing 3 times with T-TBS, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody at room temperature for 2 h. Thereafter, the membranes were washed 3 times with T-TBS, developed using enhanced chemiluminescent reagents (Amersham Life Science, Arlington Heights, IL, USA), and subjected to autoluminography for 1–5 min. Band intensities were quantified using the ImageJ software (NIH). In all instances, the membranes were stained with Ponceau S stain to verify the uniformity of protein loading and transfer efficiency across the test samples. Immunoblot with anti- β-actin (Sigma-Aldrich) was used as an internal control of protein loading when correspond. The intensity values were first normalized to β-actin or total protein and then expressed as relative protein expression, with the control lane being 1 unit.

2.5.3 Aortic cGMP levels

The thoracic aortic rings from the four groups C,T, CN and TN were excised, washed with ice-cold saline solution (0.9% wt/vol NaCl), and weighed. Aortic cGMP concentrations were measured using a cGMP ELISA kit (GE Healthcare, Pittsburgh, USA) according to the protocol of the manufacturer (acetylation enzyme immunoassay procedure for intracellular cGMP measurement).

2.5.4 NO levels in ex vivo aorta

Aorta rings from C and T rats were removed and placed in Krebs-Henseleit buffer while trimmed free of adipose and connective tissue, and then cut into 4- to 5-mm rings. Aortic rings were placed in test tubes containing 2 ml Krebs solution and with 5 μM DAF-2DA for 30 min to allow intracellular accumulation of DAF-2 and aerated with 95% O_2-5% CO_2. After that, the aortic rings were further treated with 10 nM nebivolol or vehicle control. Samples for the basal accumulation of NO were taken. Then, aortic rings were removed, dabbed dry with filter paper, and weighed. The fluorescence of the incubation solutions was measured (50 μl, in triplicate) in a 96-well microplate using a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 495 nm and an emission wavelength of 520 nm. DAF-2DA fluorescence is linearly related to the NO concentration. NO production was expressed as steady-state DAF-2DA fluorescence intensity minus its intensity before exposure to the released NO and values were normalized to tissue weight and expressed taking the control group as 1 unit [22,23].

2.5.6 NO metabolites

NO metabolites content (NOx) was determined by means of the Griess colorimetric reaction, after enzymatic reduction of nitrates to nitrites in aorta homogenates. The absorbance of the samples was determined at 540 nm, and sodium nitrate was used as a standard. Results were expressed taking the control lane as 1 unit [24].

2.6 Statistical analysis

Normal distribution of the data and the variables of the study were verified using the Kolmogorov–Smirnov test. Data were expressed as means ± SEM. Statistical analysis of drug effects on MAP, HR, western blot analysis, NO measurements was performed by two-way ANOVA. Comparison of drug interventions on LF and LVF was performed by one-way analysis of variance (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). Statistical significance was defined as $p < 0.05$.

3. Results

3.1 Temporal course of mean arterial pressure changes after intravenous administration of nebivolol

In the control group, nebivolol administration at a dose of 3 mg/kg did not modify the MAP at any time (Fig. 1A). Interestingly, in the group pretreated with the antioxidant tempol, nevibolol produced a marked decrease in MAP (Fig 1A). Conversely, vehicle did not modify blood pressure in either experimental C and T groups (data not shown).

To rule out whether the increase in the hypotensive effect of nebivolol in the tempol treated groups was due to its β-blocker adrenergic action, we used metoprolol or atenolol, two β-blocker agents lacking an effect on NO bioavailability [12]. We have previously demonstrated that nebivolol at a dose of 3 mg/kg is equivalent to a dose of 3 mg/kg of atenolol or metoprolol in regard to the MAP response [25,26]. Atenolol or metoprolol administration did not modify MAP neither in C nor T treated group (Fig. 1B). Also, to exclude whether the hypotensive effect was exclusive between tempol-third generation beta blockers association, we used N-acetyl cysteine (an antioxidant that mimics the action of glutathione) together with nebivolol. Surprisingly, in the N-acetyl cysteine group, nebivolol administration diminished MAP similar to the tempol preteated group (Fig. 1B). To address whether the hypotensive response in the TN group was related to an increase in NO level or K_{ATP} channel activation, we used L-NAME, a specific inhibitor of NOS, or glybenclamide, a K_{ATP} channel blocker. Acute preadministration of L-NAME partially blocked the hypotensive effect found in the TN group (Fig. 1A). Conversely, glybenclamide preadministration, did not modify the hypotensive response in the TN group (Fig. 1A).

3.3 Heart rate effects

Figure 2 shows the maximum response of HR changes in C and T rats after intravenous administration of nebivolol, metoprolol or atenolol. Nebivolol, metoprolol and atenolol administration induced a bradycardic response in both control and tempol groups compared to C group. However, no significant statistically difference was found between groups. Vehicle application did not modify HR in C and T experimental groups without nebivolol, metoprolol or atenolol administration (data not shown).

3.4 Effect of nebivolol on blood pressure variability

Nebivolol administration reduced VLF and LF in the TN group at 15 and 30 minutes post-administration (Table 1) with regard to control animals. However, reduction of VLF induced by nebivolol administration was not significant in the TN group compared with CN animals due to large variance of the results. Importantly, L-NAME pretreatment prevented effects on blood pressure variability demonstrating indirect evidence of the involvement of NO pathway in nebivolol-tempol synergic interaction (Table 1).

3.5 Oxidative stress parameters

3.5.1 Lipid peroxidation in aorta

In order to demonstrate that tempol exerts antioxidant effects in the normotensive C group, we performed TBARS determination as a general index of oxidative stress. As shown in Fig. 3, pretreatment with tempol produced a decrease in TBARS formation in 30% in comparison with control without antioxidant treatment indicating that tempol treatment produced an antioxidant response.

3.6 Nitric oxide pathway

3.6.1 Effects of Nebivolol on NO/cGMP levels

Nebivolol administration increased eNOS activation in the control and tempol pretreated groups. As shows in Fig. 4A, CN and TN rats showed a 1.58 and 1.68 fold higher phosphorylation of ser 1177 than C and T groups, respectively (Fig. 4A). However, no statistical difference was found between CN and TN groups. Then, we measured NO level to find whether there was any difference between the CN and TN groups. The NO levels, measured by fluorescent probe DAF-2T in aortic rings, were increased in the CN group. Interestingly in the TN group, NO fluorescence was higher than CN group (Fig 4B). Furthermore, the nitrite production measured *ex vivo* by Griess reaction was increased 3.2 folds in the TN group and 2.1 folds in the CN group with respect to C group without treatment, demonstrating an increase in NO bioavailability in the TN group compared to CN animals (Fig. 4C).

3.6.2 Effects of Nebivolol on NO/cGMP signalling in tempol treated rats

It is well know that NO exerts its biological action through the sGC enzyme. Data here reported demonstrates an increase in α and β sGC subunits in 2.1 fold expression in T group with respect to C animals (Fig. 5 A, B). In addition, cGMP, the effector of the NO pathway, was enhanced in TN when compared with CN group (Fig. 5C). It is well known that the primary downstream

target of vascular smooth muscle cGMP is PKG, which itself activates the VASP protein by phosphorylation. TN group showed a greater increase on P-VASP (ser 239) phosphorylation compared with CN (4.01 vs 2.20 fold, respectively), (Fig. 5D). This fact may be a consequence of increase in NO bioavailability together with the higher expressions of α and β guanylate cyclase found in the T and TN groups.

4. Discussion

The present study demonstrates that the combination of an antioxidant, such as tempol, and nebivolol, a NO-dependent vasodilatory beta blocker, produces a marked potentiation of nebivolol hypotensive response by enhancing NO bioavailability and the NO pathway, as a consequence of the antioxidant activity exerted by tempol. Our findings open a possible new strategy therapeutic.

We have demonstrated that nebivolol at doses of 0.3 and 3 mg/kg did not modify mean arterial pressure in normotensive conscious rats [27]. However, dual combination between tempol and nebivolol i.v administration at a dose of 3 mg/kg produces a drop in MAP without affecting HR substantially between the C, T, CN and TN groups. To address the mechanism involved in the hypotensive response, we test the possibility whether pretreatment with a NOS inhibitor, L -NAME, or with a K_{ATP} blocker, glybenclamide, could abolish this response found in the TN group. L-NAME but not glybenclamide partially blocked the drop in MAP in the TN group demonstrating that the NO pathway was involved in the hypotensive response to nebivolol in tempol pretreated rats. This last finding could be attributed to the endothelial isoform of NOS, considering that L -NAME, at the selected dose, is specific for eNOS [18].

Identification of the frequency components of blood pressure variability by power spectral analysis can potentially provide an approximation about mechanisms involved in blood pressure regulation [28-30]. In this context, it is well known that the endothelial-derived NO in rats modulates LF and VLF domains of blood pressure variability [28-30]. In fact, spectral analysis of the blood pressure recording suggests that the greater hypotensive response to nebivolol in tempol rats could be attributed to the NO pathway since nebivolol administration induced a reduction of VLF and LF only in the TN group.

On the other hand, metoprolol and atenolol, two β blockers without eNOS stimulating activity did not produce any hypotensive response in animals pretreated with tempol. All together, these findings suggest that the improvement of the NO pathway could be attributed to an increase in NO bioavailability and/or a decrease in $ONOO^-$ production. Those are beneficial effects since arterial hypertension is associated with impaired endothelium-dependent vasodilatation both in the macro and microvasculature [31,32]. Endothelium-dependent vasodilatation is mediated principally by NO and $O_2^{\cdot -}$ increased levels are associated with reduced NO bioavailability and increased $ONOO^-$. $O_2^{\cdot -}$ is constitutively produced by the vascular NAD(P)H oxidase not only in pathological situation as hypertension but also in normotensive animals. For that reason, $O_2^{\cdot -}$ seems to play a key role for the reduced endothelium-

dependent vasodilatation in arteries from normotensive and hypertensive animals leading and/or aggravating hypertension, as a consequence of eNOS uncoupling or $ONOO^-$ production [33,34]. Also, it has been shown by Oelze et al., that long-term nebivolol administration exerts antioxidant “perse” actions in an angiotensin-II dependent model of hypertension [16]. Nevertheless, in our study, the acute term of nebivolol administration is unlikely to produce neither antioxidant properties “perse” nor hypotension in the C group (Fig. 1).

In order to elucidate the mechanism of the tempol-nebivolol hypotensive effect, we studied in more detail the NO/sGC/cGMP pathway [35,36]. The two most important sites of eNOS activation are the activation at Ser 1177 and the inhibitory site Thr 495 [35, 36]. In fact, in our experimental work, nebivolol induces phosphorylation at Serine 1177 in the control and tempol treated groups. This phosphorylation at Serine 1177 was similar in CN and TN groups; indicating that nebivolol-tempol amplified response is downstream eNOS activation. To address whether the hypotensive response was due to an increase in NO bioavailability, as a consequence of $O_2^{\cdot -}$ basal levels reduction by tempol action, we evaluate NO directly by DAF and indirectly by Griess reaction. As expected, NO and nitrite levels are increased in the TN group with respect to the CN group. In the vessel wall, NO diffuses from the endothelium across cell membranes into underlying vascular smooth muscle. It is known that the well-recognized function of NO is the activation of heme containing sGC [37], which is a heterodimeric protein consisting of 73 kDa α1 and 70 kDa β1 subunits, both of which are required for catalytic activity [38,39]. sGC catalyses the conversion of guanosine-5'-triphosphate to the second messenger cGMP. In our study, we measured the cGMP levels in the four experimental groups. Consistently, TN group showed higher level in cGMP than CN group. In addition, chronic administration of tempol produced an increase in α1and β1 sGC subunits expression and this last fact may enhance the hypotensive effect of nebivolol found in the TN group. cGMP modulates numerous targets, including protein kinases such as PKG, ion channels, among others [38]. However, the primary downstream target of vascular smooth muscle cGMP is PKG, which itself activates the myosin light-chain phosphatase [37]. Taking together, the NO-cGMP pathway is a key regulator of vascular tone and that PGK mediates many of these NO/cGMP effects [39- 41].

It is well know that analysis of the phosphorylation of VASP at serine 239 is a useful biomarker of PGK activity [41, 42] and therefore NO pathway activity. Importantly, we found an increase in P-VASP at serine 239 levels higher in the TN group compared with CN animals that correlates with cGMP and NO levels. These results suggest that the over-response in the NO/cGMP pathway as a cause of the hypotensive effect in the TN group. Nonetheless, we can not discard a role of $ONOO^-$ into this effect. Since $O_2^{\cdot -}$ reacts with NO producing $ONOO^-$, another diverging mechanism could be that tempol-nebivolol association may reduce $ONOO^-$ levels preventing more eNOS declouped.

Conclusion

Up to now, there are no data available on a successful combination between antioxidant and antihypertensive drugs. Moreover, monotherapy with vitamin C or vitamin E were found to lower oxidative stress, but large scale clinical studies have not supported an effect of vitamin C or vitamin E supplementation on cardiovascular disease or mortality [43,44].

Taking together our results, we demonstrated that antioxidant therapeutic with tempol produces an enhanced hypotensive effect in nebivolol administration enhancing the NO bioavailability and sGC improving the NO/cGMP/PKG pathway. Also, into this mechanism, we can not discard a possible reduction of $ONOO^-$ levels as a consequence of less $O_2^{\cdot-}$. Since, $ONOO^-$ mediated oxidation of tetrahydrobiopterin, a critical cofactor for eNOS, which represents a pathogenic cause of uncoupling of NO synthase, tempol-nebivolol combination could be preventing this phenomenon through decreasing nitrosative stress.

To sum up, this association could lead to a new therapeutic strategy for treatment of hypertension or resistant hypertension to standard therapy. The mechanism proposed is that the reduction of $O_2^{\cdot-}$ constitutively produced by NADPH or $ONOO^-$ levels by tempol action, could enhance the NO bioavailability produced by third generation β-blocker releasing NO. Further studies are needed to establish whether the ability of this combination could attenuate hypertension in experimental models or in humans.

Acknowledgements

We are truly indebted to Dr. Monica Galleano, Dr. Cesar G. Fraga, Dr. Mariela M. Gironacci and Maria C. Litterio for their help in conducting the experiments and Diego Chiapetta for the nebivolol solution. This work was supported by grants from Secretaría de Ciencia y Técnica, Universidad de Buenos Aires, Argentina and from the Agencia Nacional de Promoción Científica (Préstamo BID PICT 00994). Ariel H Polizio, Karina B Balestrasse and Carlos A Taira are Career Investigators from CONICET, Argentina.

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Figure Legends

Fig. 1. Changes on MAP after nebivolol (3mg/kg), i.v. administration in control (CN), tempol rats (TN) and TN animals preteated with L-NAME (75 mg/kg) or glybenclamide (20 mg/kg). Changes in MAP were described as the changes in intravascular administration of nebivolol. Values are the means ± SEM of multiple experiments (n = 7, in each group). * p<0.05 versus before administration. # p<0.05 versus TN group. B) Changes on MAP after metoprolol or atenolol (3mg/kg), i.v. administration in control (C) and tempol rats (T) and changes on MAP after nebivolol (3mg/kg), i.v. administration in N-Acetyl-Cysteine preteated group. Changes in MAP were described as the changes in intravascular administration of nebivolol. Values are the means ± SEM of multiple experiments (n = 5, in each group). * p<0.05 versus before administration.

Figure 1.A

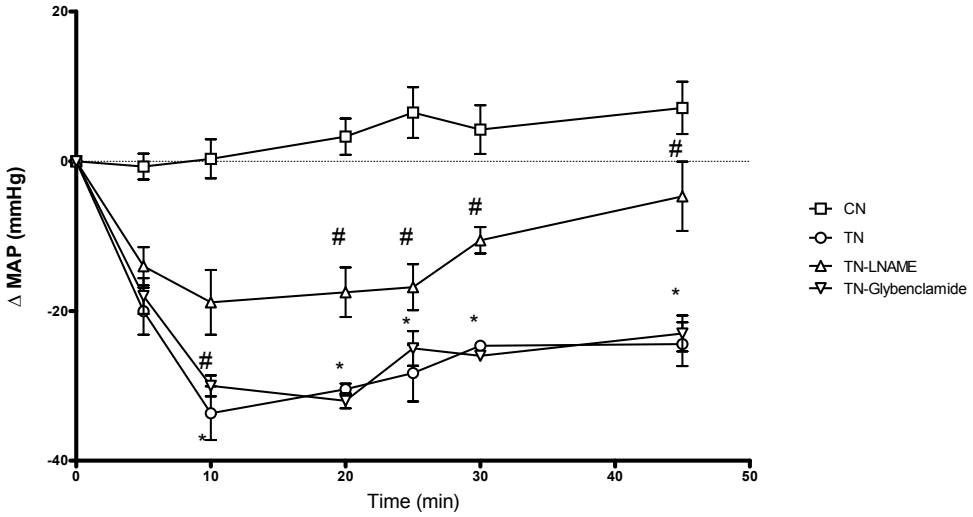

Figure 1.b

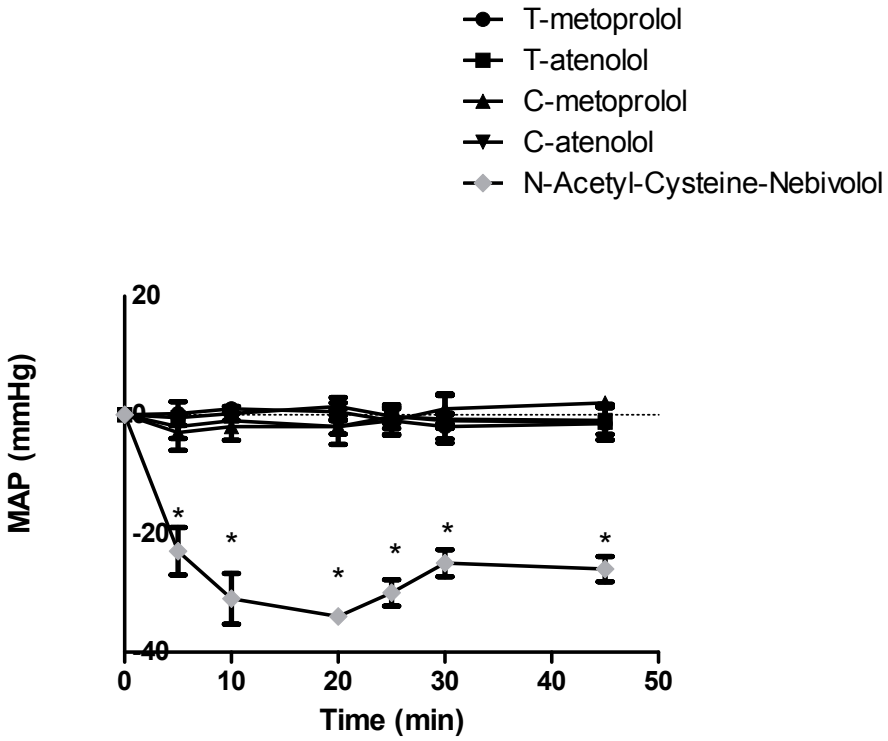

Fig. 2. Maximum changes on heart rate (HR) after nebivolol, metoprolol or atenolol (3 mg/kg), i.v. administration in control (CN), tempol rats (TN) and TN animals preteated with L-NAME (75 mg/kg). Changes in HR were described as the changes after intravascular administration of nebivolol, metoprolol or atenolol. Values are the means ± SEM of multiple experiments (n = 7, in each group). * p<0.05 versus baseline value.

Figure 2

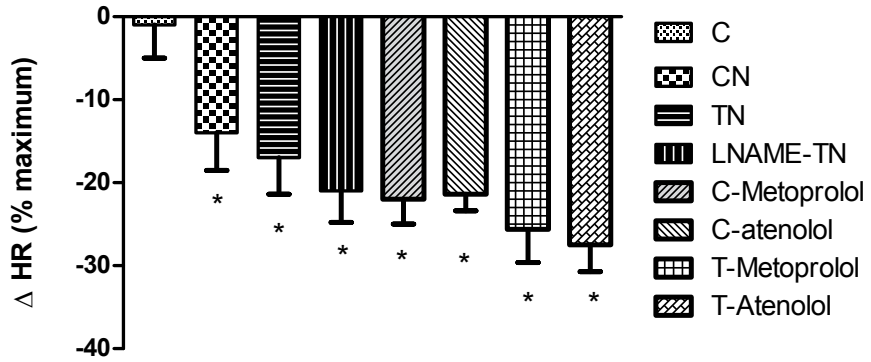

Fig. 3. **Lipid peroxidation in C and CT rats.** Bars represent mean ± S.E.M of 7 animals in each group. * p < 0.0001 tempol versus control group, n=7 each group.

Figure 3

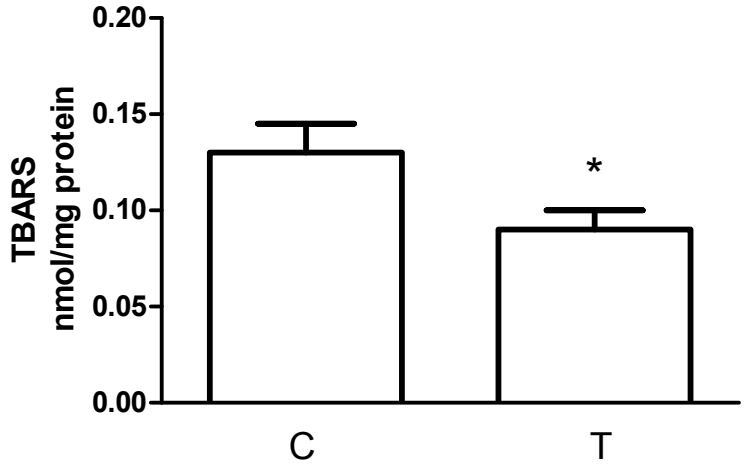

Fig. 4. (a) eNOS phosphorilation in the thoracic aortic homogenates of C,T, CN and TN after 10 minutes of nebivolol (3 mg/kg, i.v.) *in vivo* administration. Relative expression level of phospho-eNOS. Phospho eNOS was normalized using total eNOS antibody, and results are expressed as relative to a control of 1. * Significant differences (p<0.01) between nebivolol administration groups vs control group. Results show mean values of four independent measurements. b) NO measurement by fluorescence analysis of DAF-2 T (triazolofluorescein). Aortic rings from C and T groups were pretreated with nebivolol (10 μM) *ex vivo* during 10 minutes before addition of DAF-2 (5 μM). Concentrations of the product DAF-2 T were analyzed by fluorescence. Reported data are DAF-2 T concentration normalized to dryed tissue weight in each sample and expressed taking control group as 1. * P<0.05. Line (P<0.05) represents differences between CN and TN groups, n=5 each group. c) Nitrite measurement by Griess reaction. Aortic rings homogenates from C, CN, T and TN groups after 10 minutes of nebivolol (3mg/kg) i.v. *in vivo* administration. * significant differences (p<0.01) between nebivolol administration groups vs control group. Line (p<0.05) represents differences between CN and TN groups, n=7 each group. Values were normalized to protein content in each sample and results are expressed as relative to a control of 1.

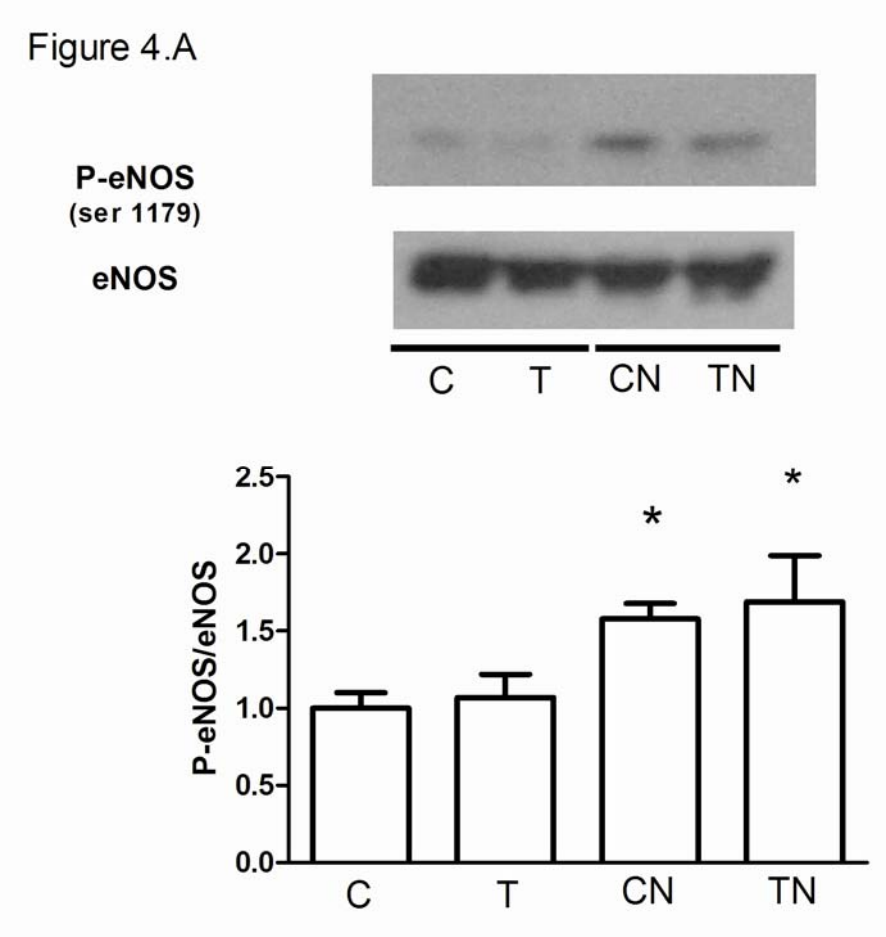

Figure 4.B

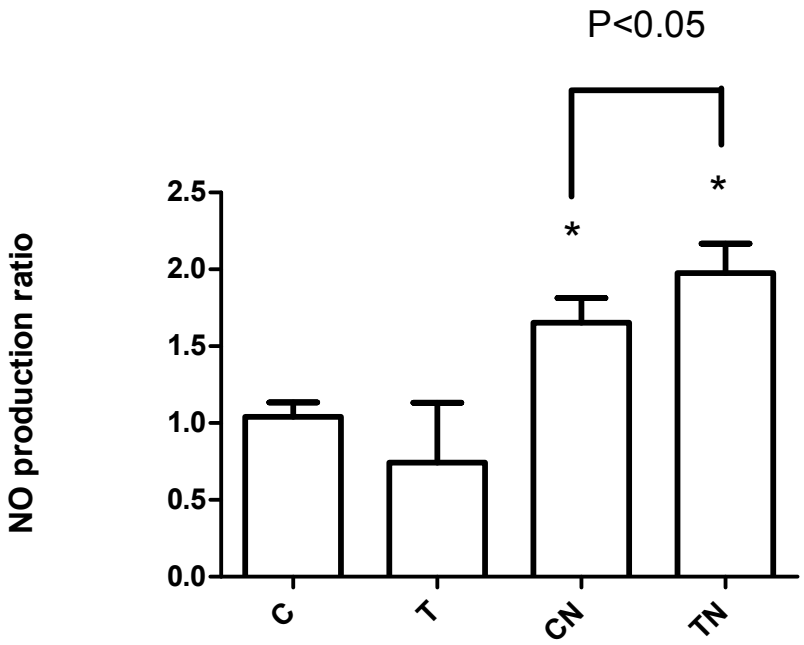

Figure 4.C P<0.05

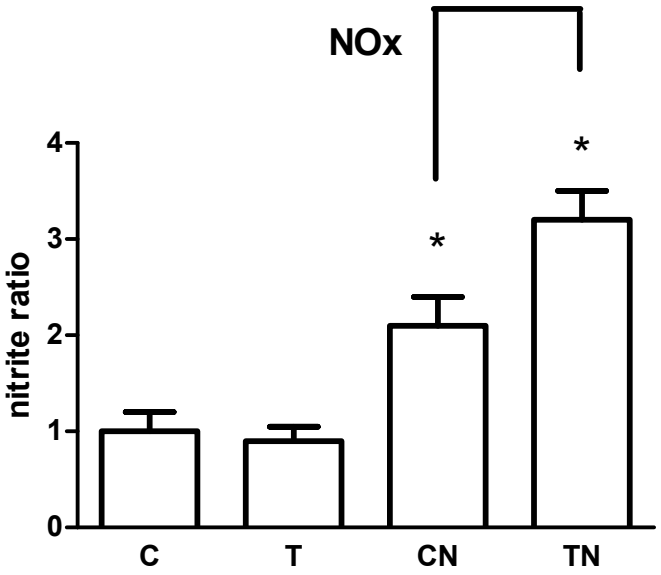

Fig. 5. **NO pathway protein expression.** Alpha (A) and beta (B) sGC protein expression in the thoracic aortic homogenates from C and T groups. Protein expression was normalized using β -actin as control antibody, and results are expressed as relative to a control of 1. * Significant differences (p<0.01) between T group vs control group. Results show mean values of four independent measurements. (c) cGMP measurement in aortic rings homogenates from C, CN, T and TN groups after 10 minutes of nebivolol (3mg/kg) i.v. *in vivo* administration. * significant differences (P<0.05) between nebivolol administration groups vs control group. Line (P<0.05) represents differences between CN and TN groups. Results show mean values of four independent measurements. (d) P-VASP (ser 239) in the thoracic aortic homogenates of C,T, CN and TN after 10 minutes of nebivolol (3 mg/kg, i.v.) *in vivo* administration. Relative expression level phospo-VASP. Phospho-VASP was normalized using beta actin antibody, and results are expressed as relative to a control of 1. * significant differences (P<0.01) between nebivolol administration groups vs control group. Line (P<0.05) represents differences between CN and TN groups. Results show mean values of four independent measurements.

Figure 5.A

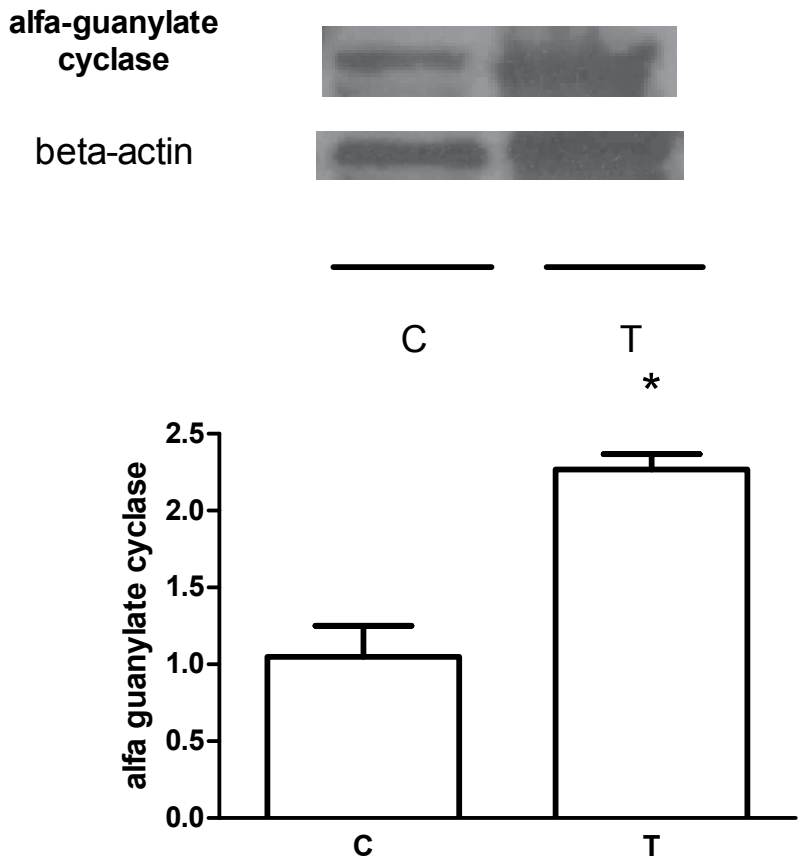

Figure 5.B

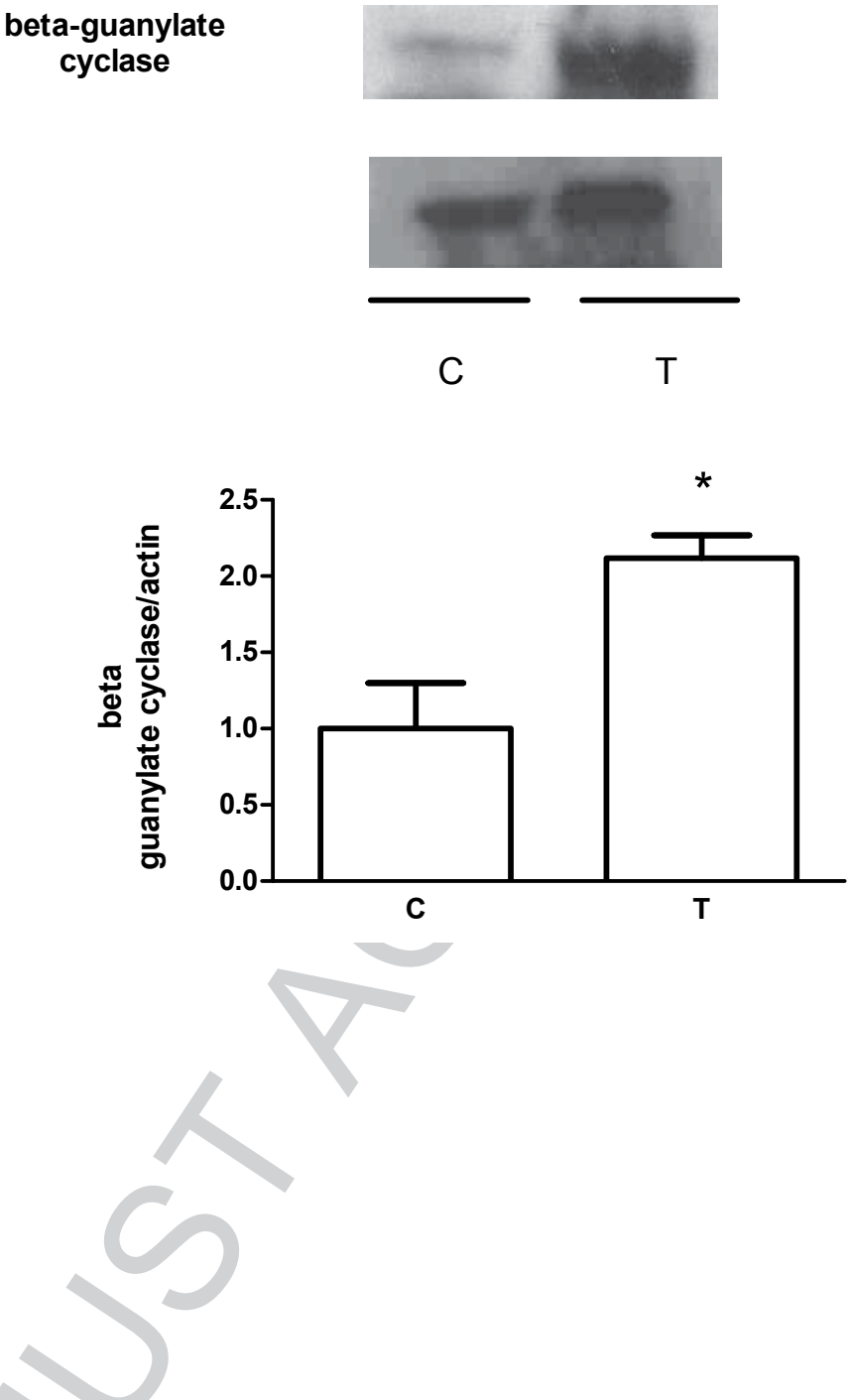

Figure 5.C

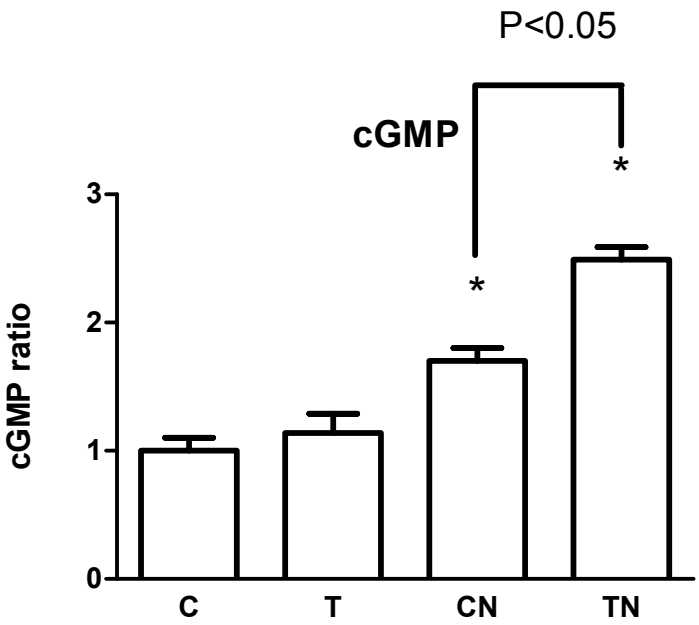

Figure 5.D

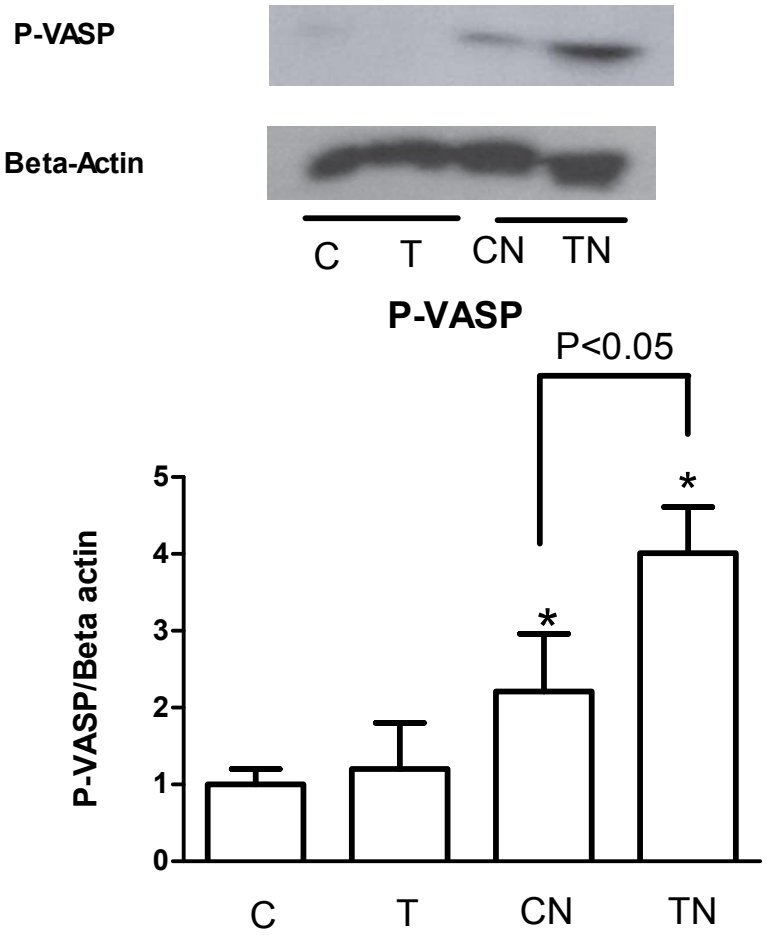

Table Legends

Table 1

Change in beat-to-beat blood pressure variability in the low frecuency (LF) and very low frequency (VLF) domain (expressed as % of baseline values) after 15 and 30 min of i.v. administration of nebivolol in control rats (CN), tempol rats (TN) and tempol rats pretreated with L-NAME (TN-LNAME). Bars represent mean ± S.E.M of 7 animals in each group. * P<0.05 vs. CN.

Parameter	CN	TN	TN-LNAME
LF variability ($mmHg_2$)			
(15 min)	91.58±12.85	48.10±4.80*	100.19±12.07
(30 min)	82.74±5.35	53.21±2.12*	99.90±7.14
VLF variability			
($mmHg_2$)	95.78±11.42	65.62±13.40	89.51±10.50
(15 min)			
(30 min)	93.78±2.44	74.48±7.41	107.42±12.53