Effect of tempol on myocardial vascular remodeling in female spontaneously hypertensive rats

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Summary. Objective: The present study evaluated whether the treatment with the superoxide anion dismutase mimetic tempol prevents the worsening in hypertension and in myocardial vascular remodeling induced by ovariectomy in female spontaneously hypertensive rats (SHR). Methods: Experiments were performed in ten week old female SHRs randomly assigned to the groups: intact (INT: given vehicle; INT+T: treated with tempol, 90 mg/kg/day), ovariectomized (OVX: vehicle and OVX+T: tempol, respectively) and ovariectomized treated with 17ß-estradiol (OVX+E2 and OVX+E2+T). Evolution of systolic blood pressure (SBP) was determined every other week in lightly restrained awake rats using a noninvasive computerized tail-cuff plethysmography system. At 18 weeks of age the heart was excised and structural changes in histopathological sections of coronary vessels were quantified on a computerized imaging system analyzer. Results: SBP was significantly lower in female SHRs treated with tempol compared to the values measured in untreated animals. In the vascular remodeling of myocardial arterioles, OVX+T rats had a lower media cross sectional area and media-to-lumen ratio than those observed in the OVX SHR. Interestingly, treatment with tempol in the presence of estradiol (in female INT and OVX+E2 SHR) increased media cross sectional area and wall-to-lumen ratio of myocardial arterioles, despite the fact that it lowered arterial pressure in those groups.
Conclusions: These results indicate that tempol prevents arterial hypertension and blunts myocardial vascular remodeling in ovariectomized SHR. Paradoxically, when tempol is given in presence of estradiol it has a detrimental effect on myocardial arteriolar remodeling.

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Introduction

Cardiovascular diseases such as hypertension and coronary artery disease are some of the most common and costly diseases in the industrialized world. Although women are largely protected from clinical coronary disease before the menopause, they rapidly develop increased risk thereafter. The incidence of cardiovascular diseases is greater in men aged 30-50 compared with women of similar age (Gerhard and Ganz, 1995; Farhat et al., 1996). Also, epidemiology studies show that women have a lower prevalence of hypertension before menopause (Isles et al., 1992), leading to the hypothesis that female hormones have significant roles in long-term regulation of arterial pressure.

Although the pathogenesis of cardiovascular diseases such as hypertension, atherosclerosis and cardiac hypertrophy is complex and multifactorial, increased reactive oxygen species (ROS) generation plays an important role (Cave et al., 2006; Paravicini and Touyz, 2008). It should be noted that there seems to be a causative relationship between increased oxidative stress and hypertension (Paravicini and Touyz, 2008) and also between oxidative stress and endothelial dysfunction (Zalba et al., 2000, 2001; Landmesser and Harrison, 2001), which often accompanies hypertension. However, the hypothesis of a parallel relationship between the progressive cessation of ovarian function and the increase of oxidative insult in hypertension still remains controversial. Several clinical trials have been performed to determine whether treatment of hypertensive individuals with antioxidants will reduce their blood pressure. The results are conflicting, with antioxidants having no effect, decreasing, or even increasing blood pressure (Palumbo et al., 2000; Kim et al., 2002;

Czernichow et al., 2005; McQueen et al., 2005; Schneider et al., 2005; Ward et al., 2007). In menopausal women, drug classes for treatment are constantly reviewed, including antioxidants (Wenger, 2008).

Human essential hypertension and animal models of hypertension are associated with hypertrophy and hyperplasia of vascular smooth muscle cells, as well as eutrophic remodeling of the existing component of the arterial wall (Heagerty et al., 1993). These vascular pathologic processes related to hypertension contribute to increased peripheral resistance in a variety of vascular beds (Heagerty et al., 1993). It is believed that in addition to neural and humoral mechanisms, local factors intrinsic to the wall of the vessels are important contributors to the regulation of peripheral resistance (Sun et al., 1995). The actions of estrogen seem to play a pivotal role in vascular biology. Estrogen replacement restores endothelium-dependent vasodilatation (Keaney et al., 1994) and modulates cell proliferation, growth and reactivity of vascular smooth muscle (Xing et al., 2009). However, the mechanisms responsible for the increase in blood pressure when levels of estradiol are low are yet to be elucidated.

We have previously shown that ovariectomy in SHR in the early hypertension stage enhanced the hypertension-associated increases of the media cross sectional area (CSA), wall-to-lumen ratio, and perivascular fibrosis in coronary microvessels to an extent similar to those found at 33 weeks of age, when hypertension and vascular remodelling were well established. In this early stage, both estrogen and its metabolite 2-methoxyestradiol administration protected the vasculature from these changes (Garcia et al., 2005; Bonacasa et al., 2008) and prevented oxidative stress in the early stages of hypertension, indicating that the effects of ovariectomy may be due to the oxidative stress likely caused by estrogen deprivation (Garcia et al., 2005, 2006; Bonacasa et al., 2008). Since oxidative stress is increased in hypertension and the treatment with anti-oxidants or agents that scavenge superoxide anion have not produced conclusive results in female animal models, the aim of the present study is to evaluate whether tempol (a superoxide anion dismutase mimetic) prevents hypertension and coronary arteriolar vascular remodeling in female spontaneously hypertensive rats (SHR) and whether it has an additional effect in the presence of estradiol, endogenously produced or administered exogenously as replacement therapy. To this end, we treated with tempol intact female Spontaneously Hypertensive Rats (SHRs), as well as ovariectomized SHRs and ovariectomized SHRs given estradiol replacement therapy during 8 weeks in the early stages of this genetic model of hypertension.

Material and methods

Experiments were performed on 10 week old female Spontaneously Hypertensive Rats (SHR) bred in the Animal Care Facility at the University of Murcia for eight weeks. Rats were chronically treated either with vehicle (tap water) or with tempol (90 mg/kg/day) in drinking water and divided into 6 groups: intact animals (INT for control and INT+T for rats given tempol); ovariectomized rats 10 week old under anesthesia with isofluorane (20% in O_2; OVX for control and OVX+T for tempol treated animals); and ovariectomized rats receiving estrogen replacement therapy with 17ß-estradiol (1.5mg every 8 weeks, subcutaneous pellet, Innovative Research of America, Sarasota, FL), as previously described (named OVX+E2 and OVX+E2+T) (Garcia et al., 2005). As previously reported (Wilcox and Pearlman, 2008), tempol was protected from light in foil-wrapped drinking bottles and the fresh tempol solution was prepared daily. All procedures performed were in accordance with the recommendations from the Declaration of Helsinki and the guiding principles in the care and use of animals approved by the American Physiological Society. All protocols were approved by the Universidad de Murcia Institutional Animal Care and Use Committee.

Protocol 1

Measurement of arterial pressure. SBP was determined once every week in lightly restrained awake rats by the tail-cuff plethysmography method (Letica Scientific Instrument) as previously described (Garcia et al., 2005). SBP was averaged from 3–5 measurements each day.

Protocol 2

Assessment of coronary vascular remodeling by histological examination. The analysis of coronary vascular remodeling was performed as described in previous studies (Garcia et al., 2005; Bonacasa et al., 2008). Hearts were flushed with saline for 10 minutes followed by a 10% formaldehyde solution for 15 minutes via retrograde infusion into the ascending aorta. The perfusion pressure of both saline and formaldehyde solutions was set and maintained similar to the arterial pressure measured in vivo in each rat. The heart was fixed in 10% buffered formaldehyde for 24 hours and conserved for a few days in sodium cacodylate solution. The left side was separated from the right one, the atria, and the great vessels. The left side was cut into seven pieces perpendicular to the long axis. After fixation and dehydration in alcohol, samples were embedded in historesin (JB-4 Polysciences, Inc. USA). Four alternated pieces were sectioned (2 μm thick) and stained with toluidine blue for remodeling analysis examination. All histopathological sections of each animal were carefully scanned using a video microscopic system made up of a microscope Olympus BX51WI coupled to a Cool-Snap digital video camera. Images were analyzed on a computer system (Zeiss KS300) calibrated with a ruled microslide. Transectional images of myocardial vessels with external diameters

from 50 to 100 μm, and from 100 to 200 μm, were studied at x100 to x200 magnifications. Only vessels with a long-to-short axis ratio of less than 1.30 were measured, thus excluding non-round vessels resulting from oblique transection or branching. Morphometric measurements of external and lumen diameters along the long and short axes of cross-sectionally cut vessels were obtained. The two measurements of external and lumen diameters were averaged. Media-to-lumen ratio and the mean for each category of vessels were calculated. The media cross-sectional area (CSA) was determined as the area of the total vessel minus the area of the total blood vessel lumen. The morphometric analysis and quantification of vascular remodeling was performed by a blinded observer.

Statistical analysis

Data are presented as mean values ± 1 SE. A two-way ANOVA for repeated measures (SBP) or a one-way ANOVA (vascular remodeling) followed by a Fisher post hoc test was used to determine the differences between and within mean values (11). A $P<0.05$ was considered to be significant.

Results

Protocol 1

Measurements of arterial pressure. A comparison of the systolic blood pressures measured in SHR animals is presented in Table 1 and Figure 1. At 10 weeks of age, SBP was similar in all groups. 4 weeks after ovariectomy, SBP was significantly higher in OVX

compared with the values measured in all other groups. Figure 1A shows how treatment with tempol in intact SHRs prevents the development of hypertension during the experiment. Figure 1B depicts that tempol treatment (and also E2 replacement therapy) prevented the notable increase of SBP seen in OVX rats. In the last 3 weeks of the study, the SBP was significantly lower in OVX+E2+T rats compared with the OVX+E2 experimental group.

Protocol 2

Assessment of coronary vascular remodeling by histological examination. The effects of tempol on coronary vascular remodeling in female SHRs are presented in figures 2 and 3. Figures 2A,B represent the results of media cross-sectional area and media-to-lumen ratio studies of myocardial vessels with diameters between 50 and 100 μm. Both parameters were significantly greater in INT+T animals (1800.27 ± 177.38 μm^2, 0.33 ± 0.04; n=11) than those measured in intact rats (1208.19 ± 86.50 μm^2, 0.25 ± 0.01; n=14). The treatment

Table 1. Changes in systolic blood pressure (SBP) measured in conscious SHR 10 to 18 week old: Intact animals (INT) and intact rats treated with tempol (INT+T).

Week of treatment	0	4	8
INT (n=14)	155.29±2.44	164.20±1.94*	166.20±1.95*
INT+T (n=14)	153.21±3.11	153.83±1.35	148.02±1.48

* indicates p<0.05 vs. INT rats. Mean values ±1 SE are presented.
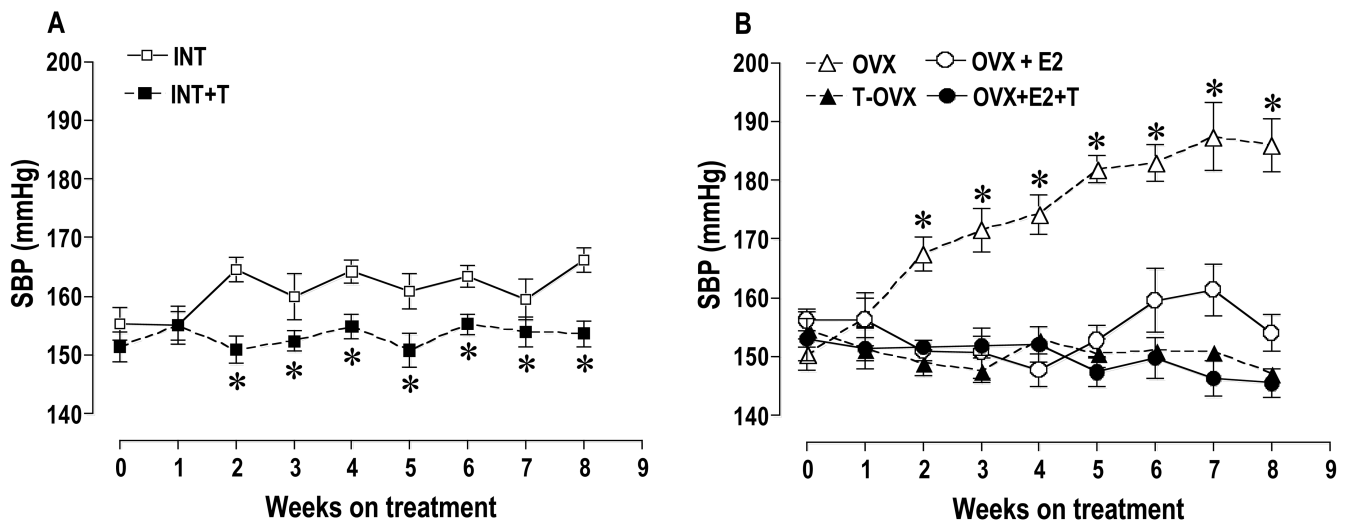

Fig. 1. Comparison of time-related changes in systolic blood pressure (SBP) measured in conscious SHRs 10 to 18 week old. **A.** Intact rats (INT) and intact rats treated with tempol (INT+T). * indicates significant difference vs. INT rats. **B.** Ovariectomized rats (OVX), ovariectomized rats treated with tempol (OVX+T), ovariectomized rats treated with 17ß-estradiol (OVX+E2) and ovarioectomized rats treated with 17ß-estradiol and tempol (OVX+E2+T) * indicates p<0.05 vs. all groups.

with tempol significantly prevented the increase of both media CSA and media-to-lumen ratio of myocardial vessels in ovariectomized animals (OVX+T, 1667.55±156.49 μm^2, 0.37±0.07; n=14) compared to those microvessels of animals treated with vehicle (OVX, 2033.82±181.72, 0.50±0.07, n=16). Notably, there is no significant difference between vascular remodeling parameters of myocardial microvessels of OVX+T and OVX+E2 (1364.01±91.89, 0.32±0.04; n=14) groups of SHR. However, the cotreatment of
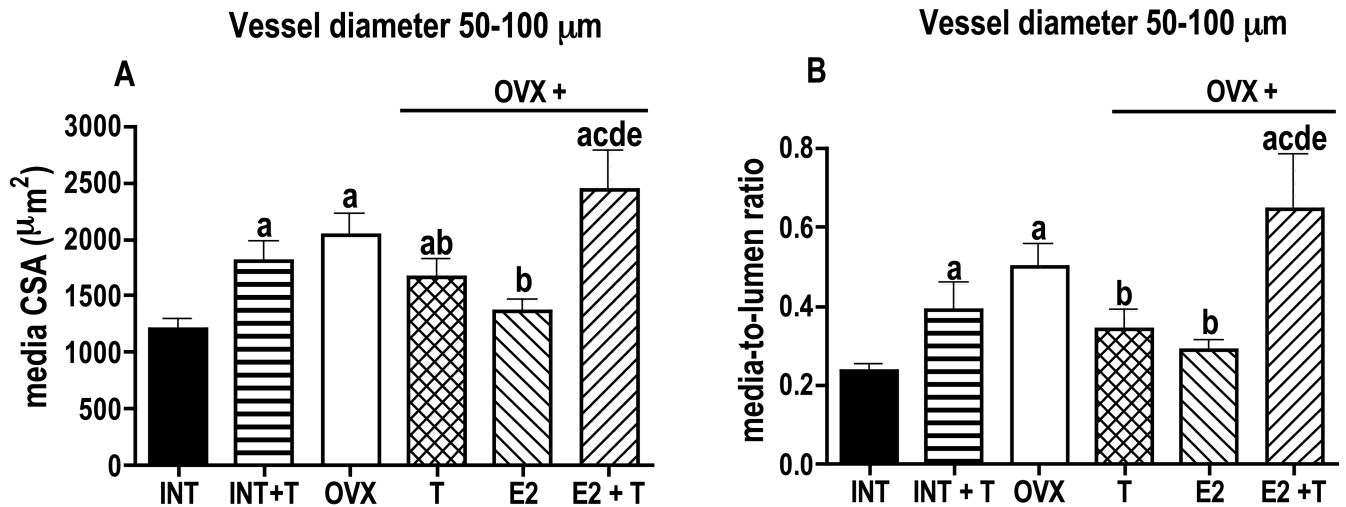

Fig. 2. Morphometry of 50-100 μm diameter myocardial vessels assessed in 18 week old female SHRs: intact rats (INT), intact rats treated with tempol (INT+T), ovariectomized rats (OVX), ovariectomized rats treated with tempol (OVX+T), ovariectomized rats treated with 17ß-estradiol (OVX+E2) and ovarioectomized rats treated with 17ß-estradiol and tempol (OVX+E2+T). Mean values±1 SE are presented. **A.** Media CSA. a indicates p<0.05 vs. INT rats, b indicates p<0.05 vs.OVX, c indicates p<0.05 vs.OVX+E2, d indicates p<0.05 vs. INT+T, and e indicates p<0.05 vs. OVX+T. **B.** Media-to-lumen ratio. a indicates p<0.05 vs. INT rats, b indicates p<0.05 vs.OVX, c indicates p<0.05 vs. OVX+E2, d indicates p<0.05 vs. INT+T, and e indicates p<0.05 vs. OVX+T.

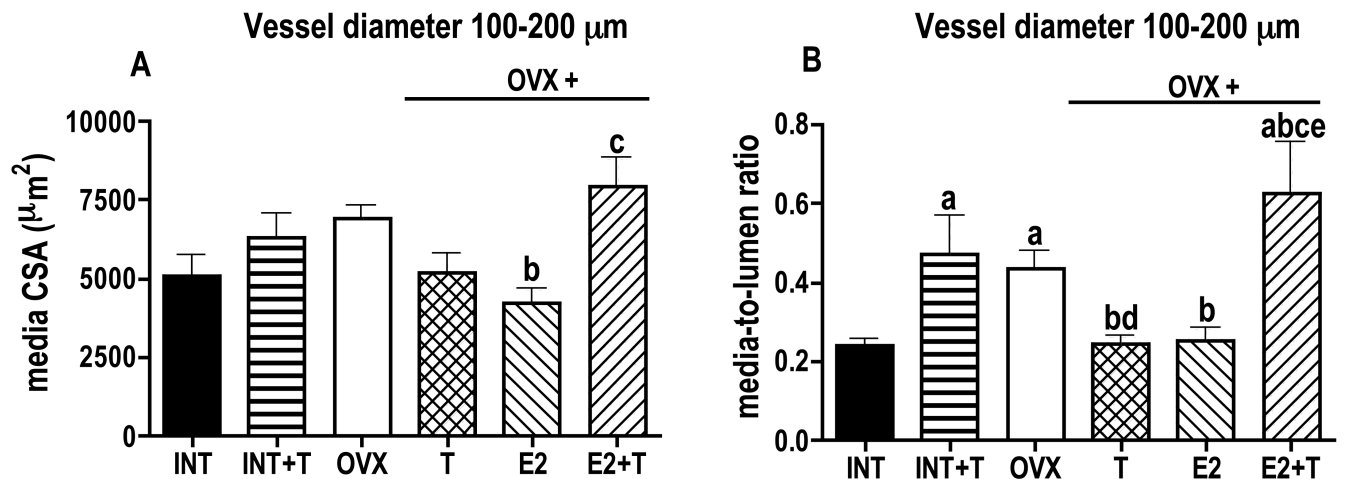**Fig. 3.** Morphometry of 100-200 μm of diameter myocardial vessels assessed in 18 week old female SHRs: intact animals (INT), intact rats treated with tempol (INT+T), ovariectomized rats (OVX), ovariectomized rats treated with tempol (OVX+T), ovariectomized rats treated with 17ß-estradiol (OVX+E2) and ovarioectomized rats treated with 17ß-estradiol and tempol (OVX+E2+T). Mean values±1 SE are presented. **A.** Media CSA values. b indicates p<0.05 vs. OVX, c indicates p<0.05 vs. OVX+E2. **B.** Media-to-lumen ratio. a indicates significant p<0.05 vs. INT rats, b indicates p<0.05 vs.OVX, c indicates p<0.05 vs. OVX+E2, d indicates p<0.05 vs. INT+T, and e indicates p<0.05 vs.T-OVX.

Table 2. Changes in systolic blood pressure (SBP) measured in conscious SHR 10 to 18 week old: ovariectomized rats (OVX), ovariectomized rats treated with tempol (OVX+T), ovariectomized rats treated with 17ß-estradiol (OVX+E2) and ovarioectomized rats treated with 17ß-estradiol and tempol (OVX+E2+T).

Week of treatment	0	4	8
OVX (n=17)	150.25±2.00	174.23±3.25*	185.99±4.43*
OVX+T (n=17)	154.69±3.03	152.90±1.06	147.19±1.24
OVX+E2 (n=10)	156.14±1.44	147.64±2.84#	153.98±3.07#
OVX+E2+T (n=14)	152.93±3.59	152.02±2.94	145.51±2.43

*: indicates $p<0.05$ vs. OVX rats. #: indicates $p<0.05$ vs. OVX+E2 rats. Mean values ±1 SE are presented.

OVX rats with tempol+E2 significantly increased both media CSA and media to lumen ratio (2434.90±347.48, 0.65±0.18; n=13). Figures 3A,B depict the myocardial vascular remodeling data of vessels with 100-200 μm of diameter. The media CSA and media to lumen ratio of INT+T animals (6272.96±782.60 μm^2, 0.47±0.10; n=12) was higher than that of intact rats (5096.28±659.74 μm^2, 0.24±0.02; n=15). Tempol prevented the increase of media CSA and media to lumen ratio of myocardial vessels in ovariectomized animals (OVX+T, 5190.55±601.75 μm^2, 0.24±0.02; n=14) compared with microvessels from untreated OVX rats (6881.36±446.78, 0.43±0.04, n=16). Similarly to the smaller vessels, there was no significant difference between vascular remodeling parameters of myocardial microvessels of OVX+T and OVX+E2 (4217.57±437.33, 0.25±0.03; n=14) groups of SHRs. However, again the cotreatment with tempol+E2 in ovariectomized animals significantly increased both media CSA and media-to-lumen ratio (7942.82±870.03, 0.62±0.13; n=11). There was no neointima formation in this experimental model.

Discussion

The major finding in the present study is that chronic treatment with tempol attenuates progression of hypertension in ovariectomized SHRs. Moreover, we demonstrate that this membrane-permeable SOD mimetic prevents vascular remodeling, a process that seems to be associated with decreased generation of vascular O_2- and increased plasma antioxidant levels. Paradoxically, treatment with tempol in the presence of estrogen (in either intact or ovariectomized+E2 rats) worsens coronary vascular remodeling despite the fact that this therapy lowers systolic pressure in those experimental groups. There are not many studies analyzing the effect of tempol in female animals, and its effect reducing BP in SHRs is particularly variable (Wilcox and Pearlman, 2008). In contrast, the effect of tempol in male SHRs is quite consistent, restoring normal blood pressure when administered by continuous infusion, intraperitoneal injection, in osmotic minipumps or in drinking water (Soule et al., 2007;

Wilcox and Pearlman, 2008). Many factors have been suggested to play a role in the increased arterial pressure in postmenopausal women; however, experimental studies are problematic because of the lack of a suitable animal model. Nonhuman primates, sheep, rabbits, mice, and rats have all been used as models of menopause with conflicting results (Thorndike and Turner, 1998; Sartori-Valinotti et al., 2007). Here we present the effect of tempol in BP in a model of spontaneous hypertension with the advantage that no other confusion factors (such as aging or uncontrollably gradual cessation of ovarian function) are present. Our group has previously described that estradiol deprivation has pro-oxidant consequences: it decreases total antioxidant status of plasma, plasma reduced-thiol levels, and plasma nitrites/nitrates concentration and increases plasma lipoperoxides (Hernandez et al., 2000). Furthermore, it is known that estradiol increases Mn-superoxide dismutase and glutathione peroxidase expression and decreases NADPH oxidase enzyme activity and supreroxide production (Lopez-Ruiz et al., 2008; Wenger, 2008). Nevertheless, some observations point at the lack of correlation between the lowering blood pressure effect of estradiol and the levels of oxidative stress (Lopez-Ruiz et al., 2008). Indeed, a consensus has not been reached over the role of exogenous or endogenous estradiol in providing protection against oxidative stress to blood vessels during hypertension. In this regard, it has been reported that in female SHRs there was no response to treatment with tempol (Fortepiani et al., 2003; Fortepiani and Reckelhoff, 2005). On the other hand, it has been described that tempol is effective in the prevention or reversal of established hypertension, although it is generally more effective in the former case, when administrated before the onset of hypertension (Wilcox and Pearlman, 2008). In our model, at an early stage of hypertension, tempol treatment succeeded in the hypothesized effect of preventing hypertension in all experimental groups.

Hypertension is associated with vascular adaptations, including altered structural properties of the arterial wall in a variety of vascular beds (Heagerty et al., 1993; Intengan and Schiffrin, 2001). The cellular processes underlying these events include hyperplasia and hypertrophy, since altered VSMC growth, migration, differentiation and increased extra cellular matrix abundance occur (Intengan and Schiffrin, 2001). Superoxide and other ROS activate multiple signaling molecules, for instance MAP kinases and so-called antioxidant proteins in the vasculature (Droge, 2002; Lassegue and Griendling, 2010). Activation of these molecules participates in cell growth, migration, expression of pro-inflammatory genes, production of extra cellular matrix proteins and contraction, which contribute to arterial remodeling in hypertension (Zalba et al., 2000; Droge, 2002; Vokurkova et al., 2007). We previously described that ovariectomy enhanced these changes induced by hypertension and that the treatment with 17ß-estradiol or 2-methoxyestradiol prevented

them, by protecting the vasculature from these changes in an early stage of hypertension (Garcia et al., 2005, 2006; Bonacasa et al., 2008), indicating therefore that the effects of ovariectomy may be due to estrogen deprivation at this point. Tempol, a reducible nitroxide, is known to have SOD mimetic activity, and the mechanism underlying its antihypertensive effect has been attributed to the blockade of the inactivation of NO by $O_2^{\bullet-}$(Schnackenberg et al., 1998). Furthermore, a correlation between Nox 1, Nox 2 and Nox 4 isoforms of NADPH oxidase expression (an important $O_2^{\bullet-}$ - releasing enzymatic complex) and hypertension has been observed repeatedly (Lassegue and Griendling, 2010), being diminished with tempol treatment (Lassegue and Clempus, 2003; Hilenski et al., 2004; Nishiyama et al., 2004; Dikalova et al., 2005). Also, in one study (Castro et al., 2009) oral administration of tempol for 8 weeks in male rats prevented the increased media:lumen ratio of the aorta and an increase in BP. In the present study, tempol treatment improved hypertrophic remodeling provoked by ovariectomy, suggesting a protective role in preventing superoxide production. In the same line of research, prolonged administration of tempol has been found to be very effective in preventing MAPK activation of several animal models of hypertension (Wilcox and Pearlman, 2008). Overall, the data in the literature indicate that tempol seems beneficial in hypertension because it lowers arterial pressure and improves arteriolar hypertrophic remodeling, in close agreement with the present data in ovariectomized SHR.

However, a new idea is evolving suggesting that a blood pressure-independent component of vascular remodeling is also present (Rigsby et al., 2007; Sakurabayashi-Kitade et al., 2009). Currently, studies in both animal models and human patients are emerging supporting this evidence. The renin-angiotensin-aldosterone system has been described as a key mediator in this pressure-independent remodeling of resistance vasculature through its effect on NADH/NADPH oxidase redox state, a major source of $O_2^{\bullet-2}$ in vascular cells (Madamanchi et al., 2005). In addition, another pressure-independent component of vascular remodeling seems to be associated with oxidative stress. In this regard, Pires et al., found that Tempol improved middle cerebral artery structure in 12-week old male Stroke Prone Spontaneously Hypertensive Rats, a chronic effect that occurred independently of blood pressure. In particular, their data suggest that superoxide is involved in arterial vessel remodeling in hypertension. On the other hand, Grassi et al. (2010) studied the alterations of subcutaneous small resistance arteries in humans with severe obesity. They recruited normotensive subjects to study vascular remodeling and endothelial dysfunction of vessels in a non-hypertensive model of high risk of cardiovascular disease. They found that media cross-sectional area and media-to-lumen ratio values were markedly and significantly greater in obese compared to lean subjects, despite the fact that the obese were normotensive. Therefore, it seems clear that factors other than arterial pressure influence arterial wall remodeling.

Unexpectedly, in the present study tempol had detrimental effects on myocardial vascular remodeling in intact rats as well as in ovariectomized animals given estradiol. Considering the normalization of arterial pressure achieved in both groups, we firstly hypothesized that a synergic beneficial effect of tempol and estradiol should take place on blood pressure and vascular remodeling, as previously reported using another antihypertensive drug (captopril) together with estradiol (Garcia et al., 2006). However, when given in the presence of estradiol, tempol clearly worsened the hypertrophic myocardial arteriolar remodeling. The mechanisms explaining this discrepancy are unknown, but one may hypothesize that the combination of two antioxidants (tempol and estradiol) may cause paradoxical accumulative effects in the redox state of cells, perhaps originating reductive stress (Dimmeler and Zeiher, 2007; Shao et al., 2012). In addition, tempol also reacts with ROS other than $O_2^{\bullet-}$ and these reactions may contribute to potentially detrimental effects (Keaney et al., 1994). Furthermore, it has been already described that at least in some experimental models estrogens may act as a pro-oxidant agent, inducing oxidative stress in the cells by a variety of mechanisms, including ROS generation, and this detrimental effect has been implicated in the pathogenesis of complications in cardiovascular disease and various types of cancer in menopausal women (Halliwell, 2000). These effects are attributed to the different role of estrogen receptors and estrogen-derived metabolites reacting with different ROS (Kumar et al., 2010). Therefore, the present results suggest that antioxidants should be used with caution in postmenopausal women on an estrogen replacement therapy until a more profound understanding of the mechanisms involved are elucidated. We are still unable to predict how a patient receiving estrogen replacement therapy will respond to an antioxidant supplementation, depending on the dose and the type of antioxidant used (Madamanchi et al., 2005).

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