Influence of ovariectomy on cardiac oxidative stress in a renovascular hypertension model

Jaqueline Barp, Carmem Luiza Sartório, Cristina Campos, Susana Francisca Llesuy, Alex Sander Araujo, and Adriane Belló-Klein

Abstract: The aim of this study was to evaluate the potential influence of endogenous ovarian hormones on cardiac oxidative stress in renovascular hypertension. Female Wistar rats ($N = 10$ per group) were divided among 4 groups: (*i*) normotensive control; (*ii*) hypertensive control; (*iii*) normotensive ovariectomized; and (*iv*) hypertensive ovariectomized rats. To induce hypertension, 2-kidney 1-clip (2K1C) Goldblatt's method was followed. Blood pressure (BP) was enhanced (25%) in 2K1C and it was not further altered in hypertensive ovariectomized animals. Lipid peroxidation (measured by thiobarbituric acid reactive substances; TBARS) increased in heart homogenates after ovariectomy (253%) and was additionally augmented when associated with hypertension (by 28%). Superoxide dismutase and catalase activities were similar in both hypertensive groups. Hypertension enhanced glutathione peroxidase activity (75%), but the association with ovariectomy prevented this change. Total radical trapping antioxidant potential (TRAP) decreased in hypertensive rats (34%) and was recovered when associated with ovariectomy. However, this adaptation seems not to be sufficient to avoid the increased oxidative damage in ovariectomized hypertensive animals. These results suggest a protective role for physiological ovarian hormones in the cardiac oxidative stress induced by 2K1C hypertension.

Key words: 2-kidney 1-clip, renovascular hypertension, oxidative stress, antioxidant enzymes, heart, ovarian hormones, estrogen, ovariectomy.

Résumé : Le but de cette étude était d'évaluer l'influence potentielle des hormones ovariennes endogènes sur le stress oxydant cardiaque dans l'hypertension rénovasculaire. Des rats femelles Wistar ($N = 10$ par groupe) ont été divisées en quatre groupes : (*i*) contrôle normotendu; (*ii*) contrôle hypertendu; (*iii*) normotendu ovariectomisé et (*iv*) hypertendu ovariectomisé. La méthode de Goldblatt 2R1C (2-reins/1-clip) a été utilisée afin d'induire l'hypertension. La pression sanguine était augmentée (25 %) chez les animaux 2R1C et elle n'était pas davantage affectée chez les animaux hypertendus ovariectomisés. La peroxydation des lipides était augmentée dans les homogénats de cœur après l'ovariectomie (253 %) et elle était davantage accrue par l'hypertension (de 28 %). Les activités de la superoxyde dismutase et de la catalase étaient similaires dans les deux groupes hypertendus. L'hypertension augmentait l'activité de la glutathion peroxydase (75 %), mais l'ovariectomie prévenait ce changement. Le potentiel antioxydant total de capture de radicaux était diminué chez les rats hypertendus (34 %) mais il était rétabli par l'ovariectomie. Cependant, cette adaptation ne semble pas suffisante pour empêcher l'augmentation des dommages oxydants chez les animaux hypertendus ovariectomisés. Ces résultats suggèrent que les hormones ovariennes physiologiques jouent un rôle protecteur dans le stress oxydant cardiaque induit par l'hypertension 2R1C.

Mots-clés : 2-reins/1-clip, hypertension rénovasculaire, stress oxydant, enzymes antioxydantes, cœur, hormones ovariennes, estrogène, ovariectomie.

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Introduction

Reduction in ovarian hormone levels has been associated with many unfavorable cardiovascular outcomes, such as lipid profile changes, coagulation disorders, altered vascular reactivity, and endothelial dysfunction (Matthews et al. 1989). Thus, menopause may be implicated in the pathophysiology of many cardiovascular diseases, such as hypertension (Stramba-Badiale et al. 2006). According to Mendelsohn and Karas (2005), premenopausal women have less cardiovascular disease than men at the same age. Nevertheless, these cardioprotective effects disappear after menopause (Stramba-Badiale et al. 2006). In this scenario, estrogen replacement may have some benefit, being involved in blood pressure attenuation (Hazzard 1989). However, large trial results have demonstrated that estrogen replacement in women with documented coronary heart disease does not reduce cardiovascular disease outcomes (Grady et al. 2002).

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J. Barp, C.L. Sartório, C. Campos, S.F. Llesuy, A.S. Araujo, and A. Belló-Klein. Cardiovascular Physiology Laboratory, Federal University of Rio Grande do Sul (UFRGS), Avenida Sarmento Leite 500, CEP 90050170, Porto Alegre, Brazil.

Corresponding author: Adriane Belló-Klein (e-mail: belklein@ufrgs.br).

This article is one of a number of papers published in the Special Issue entitled "Heart Health and Care," which focuses on new knowledge of the physiology of cardiovascular functions in health, and pathophysiology of cardiovascular dysfunctions.

Renovascular hypertension is one of the most common forms of secondary hypertension and progressive loss of renal function (Garovic et al. 2005). Renovascular hypertension develops earlier in women than men, the main etiologic factors being fibromuscular dysplasia and atherosclerotic disease, respectively (Elliott 2008). The Goldblatt 2-kidney 1-clip (2K1C) hypertension model is a long-established model in the study of renovascular hypertension (Min et al. 2005). In this model, experimental data show that the renal hypertension induction rate is higher in males than in (intact) females (Okuniewski et al. 1998). These data indicate that the establishment of renovascular hypertension is gender-dependent. Previous data from our group demonstrated that sex hormone changes may affect myocardial oxidative stress in rats, showing protection associated with estrogens but not with testosterone (Barp et al. 2002).

Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and their metabolization by antioxidant systems (Oliveira-Sales et al. 2008). Under normal conditions, non-enzymatic (Lissi et al. 1995) and enzymatic (Lenfant et al. 2003) antioxidant defenses may be able to prevent oxidative stress. The antioxidant enzyme superoxide dismutase (SOD) removes superoxide anions by converting them to hydrogen peroxide (Singal et al. 1988). Subsequently, glutathione peroxidase (GPx) and catalase (CAT) convert hydrogen peroxide to water (Lenfant et al. 2003). Estrogen may be considered as a non-enzymatic antioxidant because it may act as a ROS scavenger through its phenolic group (Massafra et al. 1998). In fact, estrogen protects cardiac muscle from oxidative damage through its antioxidant activity (Persky et al. 2000). This effect may also be accomplished by its capacity for inducing the upregulation of antioxidant defense mechanisms, and by the induction of antioxidant enzyme expression (Suzuki et al. 2006).

Amirkhizi et al. (2010) found that antioxidant enzyme activities are decreased in hypertensive women, leading to oxidative stress. Moreover, Belló-Klein et al. (2001) also found impaired total radical-trapping antioxidant potential (TRAP) in the myocardium of hypertensive rats. In hypertension, oxidative stress is also due to enhanced ROS production (Touyz 2004). Thus, there is much evidence that oxidative stress is implicated in the pathophysiology of hypertension, which is one of the major risk factors for cardiovascular outcomes (Rueckschloss et al. 2003). Moreover, increased systemic oxidative stress is involved in the pathogenesis of renovascular hypertension (Lerman et al. 2001; Kao et al. 2010).

However, there is a lack of information about the role of ovarian hormones in cardiac oxidative stress in renovascular hypertension. Therefore, the purpose of the present study was to evaluate the influence of ovarian hormones withdrawal on cardiac oxidative stress in 2K1C hypertensive female rats.

Methods

Animal groups and surgical procedures

The experiments were approved by the institutional animal care unit, and conducted in accordance with the principles and guidelines of Research Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

Female rats (200–250 g, $N = 40$) were provided by the animal facility of the Federal University of Rio Grande do Sul. To induce renovascular hypertension, the classical technique of Goldblatt was followed (Goldblatt et al. 1934). All rats were anesthetized with ketamine hydrochloride (90 mg·(kg body mass)$^{-1}$ intraperitoneal injection (i.p.)) and xylazine (10 mg·kg^{-1}, i.p.). The hypertensive group of rats received a silver clip with a 300 μm gap width on the left renal artery. Control rats were also anesthetized, and the left renal artery was isolated in the same manner as for the hypertensive rats, but without applying the clip.

Ovariectomy was performed immediately after renal clamping surgery. In brief, a small abdominal incision was made and the ovaries were located. A silk thread was tightly tied around the oviduct, including the ovarian blood vessels. The oviduct was sectioned and the ovaries removed. The skin and muscle wall were then sutured with silk thread. In sham operated controls, same procedure was performed except for removal of the ovaries. After surgery, the animals received an injection of antibiotics (40 000 U·(kg penicillin G procaine)$^{-1}$, by intramuscular injection (i.m.)) (Hernández et al. 2000). All of the females test rats were in the estrous phase at the time of surgery. According to the surgical treatment, animals were divided among 4 groups: (*i*) normotensive control; (*ii*) hypertensive control; (*iii*) normotensive ovariectomized; and (*iv*) hypertensive ovariectomized rats. All animals had access to food and water, ad libitum.

Cardiovascular evaluations

Catheter implantation was performed 20 days after induction of hypertension and ovariectomy, and 24 h before the blood pressure was measured. Rats were anesthetized with ketamine hydrochloride (90 mg·kg^{-1} i.p.; König Lab S.A., São Paulo, Brazil) and xylazine (10 mg·kg^{-1} i.p.; Virbac do Brasil I.P., S.P.). A catheter (PE-10) filled with saline was implanted in the carotid artery and attached to PE-90 tubing connected to a strain-gauge transducer (Narco Bio-Systems Miniature Pressure Transducer RP 1500) for direct measurements of mean arterial pressure (MAP) and heart rate (HR). MAP and HR signals were recorded for 15 min (CODAS, 1 kHz; Dataq Instruments, Akron, Ohio, USA). After the cardiovascular evaluations, rats were killed by decapitation. The blood was collected for hormonal measurements and the hearts were removed for posterior biochemical analysis.

Hormonal measurements

Plasma 17β estradiol was measured 21 days after surgery by radioimmunoassay using a Biomedical kit (Biomedical Technologies, Inc., Stoughton, Massachussetts, USA).

Thiobarbituric acid reactive substances

Absorbance measurements at 535 nm were used to measure the reaction between thiobarbituric acid and the lipid peroxidation products, resulting in the formation of a chromogen (Schiff's base). The results were reported in nanomoles per milligram of protein. Commercially available malonaldehyde was used as the standard (Buege and Aust 1978).

Total radical-trapping antioxidant potential

TRAP, which indicates the total antioxidant capacity present in a homogenate, was measured by chemiluminescence using 2,2'-azo-bis(2-amidinopropane) (ABAP, a source of alkyl peroxyl free radicals) and luminol. A mixture consisting of

20 mmol·L^{-1} ABAP, 40 μmol·L^{-1} luminol, and 50 mmol·L^{-1} phosphate buffer (pH = 7.4) was incubated to achieve a steady-state luminescence from the free radical-mediated luminol oxidation. A calibration curve was obtained by using different concentrations (between 0.2 and 1 μmol·L^{-1}) of Trolox (hydrosoluble vitamin E) (Evelson et al. 2001). Luminescence was measured in a liquid scintillation counter using the out-of-coincidence mode and the results were expressed in millimoles per litre of Trolox.

Antioxidant enzyme activities

SOD activity was determined in heart homogenates and is expressed as units per milligram of protein. This determination was based on the inhibition of superoxide radical reaction with pyrogallol (Marklund 1985). CAT activity was determined in heart homogenates by following the decrease in 240 nm absorption of hydrogen peroxide. It is expressed in picomoles per milligram of protein (Aebi 1984).

GPx activity was measured in heart homogenates by monitoring NADPH oxidation at 340 nm. Glutathione peroxidase activity is reported in nanomoles per minute per milligram of protein (Flohé and Gunzler 1984).

Protein quantification

Protein concentration was measured by the Lowry method (Lowry et al. 1951) using bovine serum albumin as the standard.

Statistical analysis

The data are expressed as the mean ± SD. One-way ANOVA with Student–Newmann–Keuls post hoc test was used to test the interaction from normotensive ovariectomized compared with hypertensive ovariectomized rats. A value for $P < 0.05$ is considered statistically significant.

Results

Heart rate did not differ among the groups (data not shown). To confirm the success of our model of hypertension, MAP was measured. MAP was significantly enhanced by inducing hypertension. Of the animals submitted to 2K1C surgery, 15% did not develop hypertension, and were thus excluded from the study. MAP was 25% higher in the hypertensive control group ($P < 0.05$) and 31% higher in hypertensive ovariectomized group ($P < 0.05$), compared with their respective controls. No significant difference was found in MAP between hypertensive control and hypertensive ovariectomized groups (Fig. 1).

To verify the efficacy of ovariectomy, hormonal quantification of 17β-estradiol was performed in plasma samples 21 days after surgery. The concentration of 17β-estradiol (in pg·mL^{-1}) was reduced by about 96% ($P < 0.05$) in ovariectomized rats (28 ± 3) compared with the respective controls (690 ± 52).

Tecidual lipid peroxidation measurement by thiobarbituric acid reactive substances (TBARS) was strongly augmented (253%) after ovariectomy (Fig. 2A). Ovariectomy showed significant interaction with hypertension, as observed in the additional increase (28%) in TBARS levels from hypertensive ovariectomized compared with normotensive ovariectomized rats (P <0.01).

Fig. 1. Mean arterial pressure (MAP) at 21 days after ovariectomy and 2-kidney 1-clip (2K1C) surgery. *, $P < 0.01$. Data are expressed as the mean ± SD of 10 female rats per group; 1 mm Hg = 133.322 Pa.

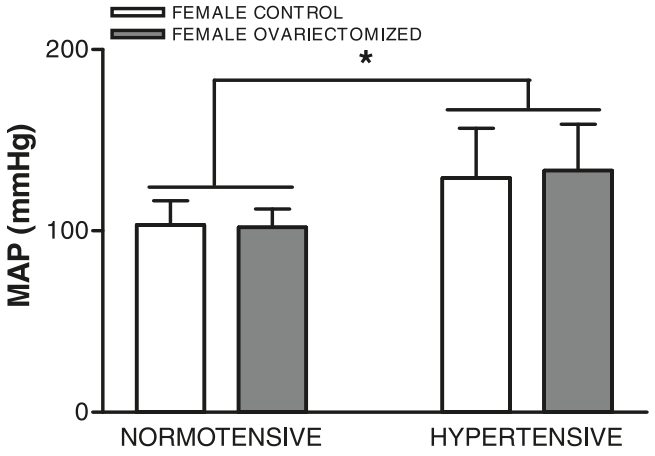
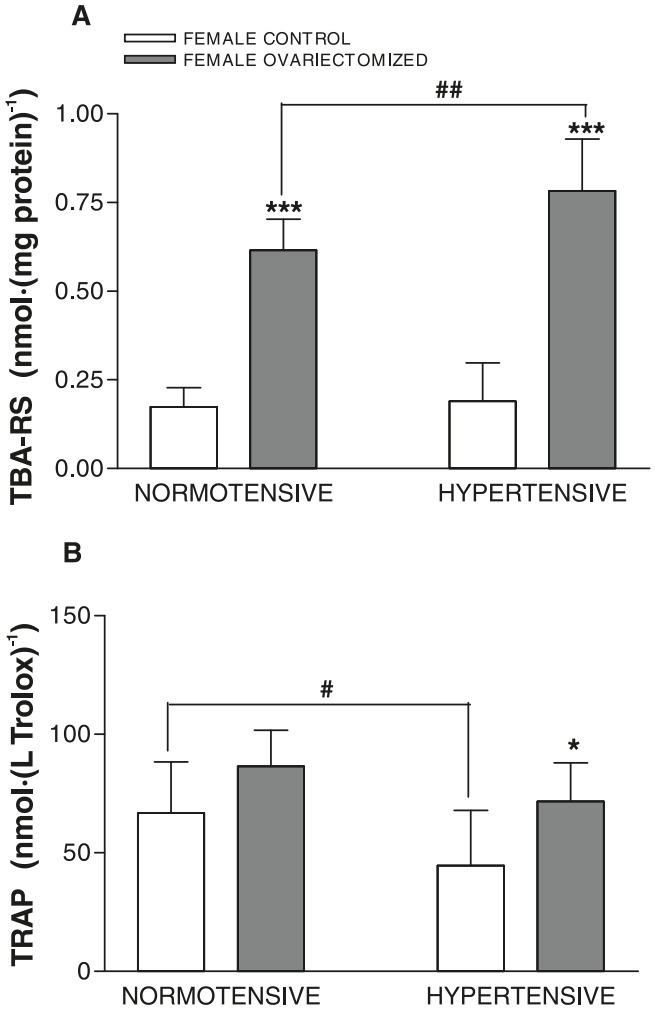


Fig. 2. (A) Thiobarbituric acid reactive substance (TBARS) levels and (B) total radical trapping antioxidant potential (TRAP) in heart homogenates. *, $P < 0.05$; ***, $P < 0.001$ compared with the respective controls; ##, $P < 0.01$; #, $P < 0.05$. Data are expressed as the mean ± SD of 10 female rats per group.



Ovariectomy had no significant influence on TRAP measurement in the normotensive group. Nevertheless, hypertensive control animals had lower TRAP levels ($P < 0.05$) compared with normotensive control and hypertensive ovariectomized rats (by 34% and 36%, respectively). In hypertensive ovariectomized rats, TRAP levels did not differ from the normotensive animals (Fig. 2B).

SOD activity was enhanced by hypertension in both groups ($P < 0.05$) by approximately 44% (Fig. 3A). Ovariectomy did not significantly alter this parameter.

CAT had its activity elevated (~95%) by ovariectomy ($P < 0.001$) and by hypertension (~39%) ($P < 0.05$) when compared with their respective control groups. Hypertension interacted with ovariectomy, preventing CAT activity increase in ovariectomized animals (Fig. 3B).

GPx activity was markedly increased by hypertension (~75%) in control animals ($P < 0.001$) (Fig. 3C). However, this enhancement was not observed in ovariectomized animals when associated with hypertension.

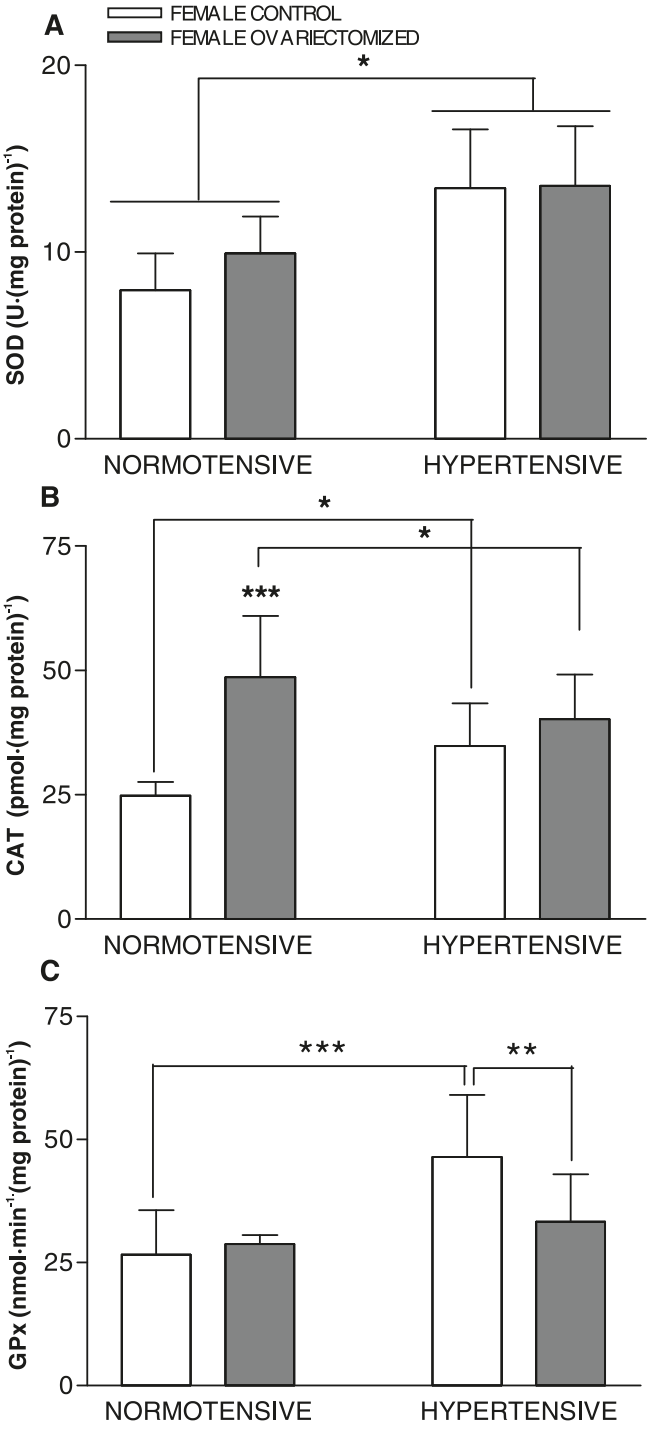
Discussion

The most relevant finding of this study is that in the presence of ovarian hormones in physiological levels, enzymatic antioxidant defenses were induced by hypertension in such a way that no cardiac lipid peroxidation was noticed. However, ovarian hormone withdrawal associated with hypertension resulted in a distinct answer profile, favoring the non-enzymatic antioxidants, which was not enough to avoid the appearance of oxidative damage.

As expected, increased blood pressure was observed in 2K1C rat hypertension model. Our results are in agreement with Mendonça et al. (2007), who also demonstrated that in rats, renovascular hypertension enhances blood pressure, per se. Additionally, our results did not demonstrate that ovariectomy exerts any influence in this parameter. In agreement with our results, Okuniewski et al. (1998) demonstrated, in the same experimental model, that blood pressure was not further incremented by ovarian hormone withdrawal.

The augmented arterial blood pressure found in 2K1C was not associated with changes in cardiac oxidative damage in female control rats. However, hypertension has induced an adaptation by antioxidant enzymes, which could be due to an increased ROS production. It is well documented that the formation of superoxide anion ($O_2^{\bullet -}$) by the endothelial gp91phox-containing NADPH oxidase is increased in the 2K1C model and contributes to the development of renovascular hypertension (Jung et al. 2004). Increased $O_2^{\bullet -}$ levels would contribute to the higher SOD activity found in the present study, which could lead to an increased hydrogen peroxide (H_2O_2) concentration. The conversion of H_2O_2 to water may be catalyzed by CAT and GPx (Lenfant et al. 2003). Our results indicate that there was an increase in CAT and GPx activity in hypertensive rats. Consistent with these findings, there was an increase in plasma GPx activity in hypertensive patients (Simic et al. 2006). This enzyme is central to peroxide metabolism in heart tissue (Kaul et al. 1993). In this process, GPx utilizes reduced glutathione (GSH) as a substrate, leading to a reduction in the levels of this important non-enzymatic antioxidant defense. One adequate method to evaluate this defense is through measuring

Fig. 3. (A) Superoxide dismutase (SOD); (B) catalase (CAT) and (C) glutathione peroxidase (GPx) activities in heart homogenates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Mean ± SD.



TRAP, which indicates the levels of mostly non-enzymatic hydrophilic antioxidants, such as glutathione and vitamin C (Lissi et al. 1995). In fact, in female hypertensive rats, a decrease in TRAP was observed. Furthermore, in male hypertensive rats reduced TRAP levels were associated to cardiac oxidative damage (Belló-Klein et al. 2001). Previous observations of other studies in male renovascular hypertensive rats also showed an increase in oxidative damage (Lerman et

al. 2001; Agarwal et al. 2004), reinforcing the protective role of the endogenous ovarian hormones.

Considering the cardioprotection promoted by ovarian hormones, their reduction would contribute to increased oxidative damage. The present study showed a significant increase in TBARS after ovariectomy. This data corroborates previous work from our laboratory (Barp et al. 2002) that demonstrated ovariectomy promotes increased myocardial oxidative damage. The present study also demonstrated that withdrawal of ovarian hormones causes an increase in CAT activity. As 17β-estradiol has intrinsic antioxidant properties (Ruiz-Larrea et al. 2000), its lack results in enhanced CAT activity, probably due to an increased concentration of hydrogen peroxide. Our results are in agreement with Konyalioglu et al. (2007), who also demonstrated an increased cardiac CAT activity in ovariectomized rats. We observed no differences in SOD and GPx activity in either of the normotensive groups. Likewise, findings from our laboratory also reported that ovariectomy did not induce GPx activity in female rat heart (Barp et al. 2002). Non-enzymatic defenses (TRAP levels) were also not altered by ovariectomy.

In the absence of physiological ovarian hormones, induced hypertension further augmented oxidative stress. In this situation, antioxidant enzymes did not show any incremental activity, and even GPx is depressed. This last effect may reflect the capacity of estrogen for inducing antioxidant enzymes, as was previously described (Suzuki et al. 2006). Moreover, decreased GPx activity would promote a preservation of GSH, which would lead to increased TRAP levels when ovariectomy was associated with hypertension. Despite this adaptive response, it was not sufficient to avoid the increment in oxidative stress in hypertensive ovariectomized rats, probably because antioxidant enzyme response did not follow the same pattern of those non-ovariectomized hypertensive rats. The additive effects of hypertension over ovariectomy prevented an enzymatic adaptive response, which could be related to oxidative inhibition (Jacob et al. 2009), since oxidative stress is further enhanced in this group.

According to our knowledge, this is the first report in the literature that shows a protective role for endogenous ovarian hormones in cardiac oxidative stress induced by 2K1C hypertension. The main adaptive changes promoted by hypertension are dependent on these hormones and include an induction of antioxidant enzymes. However, the association of hypertension and ovariectomy resulted in non-enzymatic antioxidant adaptation, but not enough to avoid oxidative damage. These findings support the design of further studies to explore the mechanisms involved in these adaptive reactions.

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