Renovascular Hypertension Leads to DNA Damage and Apoptosis in Bone Marrow Cells

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Angiotensin II (Ang II), which plays a pivotal role in the pathophysiology of the two-kidney, one-clip (2K1C) Goldblatt hypertension, has been associated with augmented generation of reactive oxygen species (ROS) in some cells and tissues. In the present study, we evaluated the influence of 2K1C hypertension on oxidative stress, DNA fragmentation, and apoptosis of bone marrow (BM) cells. Two weeks after the renal artery clipping or Sham operation, flow cytometry analysis showed a higher production of superoxide anions (approximately sixfold) and hydrogen peroxide (approximately twofold) in 2K1C hypertensive than in Sham normotensive mice. 2K1C mice also showed an augmented DNA fragmentation (54%) and apoptotic cells (21%). Our data show that the 2K1C renovascular hypertension is characterized by an increased production of ROS, DNA damage, and apoptosis of BM, which is a fundamental source of the cells involved in tissue repair.

Introduction

IN RENOVASCULAR HYPERTENSION, the most common form of secondary hypertension (Missouris *et al.*, 1998; Uzu *et al.*, 2002), the reduction of renal blood flow induces excessive activation of the renin-angiotensin system (RAS) (Campagnaro *et al.*, 2012). In the two-kidney, one-clip (2K1C) Goldblatt animal model, hypertension is induced by unilateral renal artery stenosis leading to augmented plasma angiotensin II (Ang II) concentration (Campagnaro *et al.*, 2012; Nogueira *et al.*, 2012), which plays an important role in the pathogenesis of renovascular hypertension. Moreover, this peptide exerts an essential contribution in controlling several physiological functions in different organs, once it can be locally synthesized for control of local functions (Cheng *et al.*, 1995; Cohn and Tognoni, 2001; Ruiz-Ortega *et al.*, 2001).

Abundant evidence demonstrates that increased oxidative stress results from an imbalance between the production and inactivation of reactive oxygen species (ROS) by antioxidant defense mechanisms (Oliveira-Sales *et al.*, 2008). In addition, it is well known that ROS affects the entire RAS signaling in bone marrow (BM) cells (Ruiz-Ortega *et al.*, 2001; Ceron *et al.*, 2010), which are known to participate in tissue repair (Cuende *et al.*, 2012).

Although BM cells play an important role in hypertensive-induced diseases (Wagner *et al.*, 2012) and that 2K1C hypertension leads to increased production of ROS (Cam-

pagnaro *et al.*, 2013), the impact of this renovascular hypertension on apoptosis in BM cells has not yet been investigated in this murine model. Therefore, the present study was designed to test the hypothesis that the augmented production of superoxide anions ($\bullet\text{O}_2^-$) and hydrogen peroxide (H_2O_2) leads to DNA fragmentation and apoptosis in the 2K1C hypertension model.

Materials and Methods

Animals

Experiments were performed in male C57BL/6 (C57, $n=26$) mice weighing 23 g in average, randomly distributed into two groups: sham and 2K1C. Animals were bred and maintained in the animal care facilities of the Laboratory of Transgenes and Cardiovascular Control in the Health Sciences Center at the Federal University of Espirito Santo, Brazil. They were fed a standard chow diet and provided water *ad libitum*. Animals were housed in individual plastic cages with a controlled temperature (22°C–23°C) and humidity (60%) and were exposed to a 12-h light/12-h dark cycle. All experimental procedures were performed in accordance with the guidelines for the care and handling of laboratory animals as recommended by the National Institutes of Health (NIH), and study protocols were previously approved by the Institutional Animal Care and Use Committee (CEUA-Emescam, Protocol No. 010/2009).

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Induction of 2K1C renovascular hypertension

We used a mouse model of 2K1C angiotensin-dependent hypertension as previously described (Nogueira *et al.*, 2007, 2012; Campagnaro *et al.*, 2013). Briefly, the animal was anesthetized (ketamine/xylazine 91/9.1 mg/kg, i.p.) and the left renal artery was exposed through a retroperitoneal flank incision and carefully isolated from the renal vein, nerves, and connective tissues. A stainless steel clip (0.12 mm opening width) was placed around the renal artery close to the abdominal aorta, resulting in partial occlusion of renal perfusion. The animal received a single injection of benzylpenicillin benzathine (7 mg/kg, i.m.), and was allowed to recover under care for 24 h. Control mice underwent the same surgical procedure except for placement of the renal artery clip (sham).

Hemodynamic measurements

Two weeks after the renal artery clipping or sham operation, the animals were anesthetized with a combination of ketamine/xylazine (91/9.1 mg/kg, i.p.) and a catheter (0.040 mm OD $\times$ 0.025 mm ID; Micro-Renathane; Braintree Science) was filled with a heparin saline solution and inserted into the right carotid artery. Then, the catheter was tunneled subcutaneously and brought out at the nape of the neck. Immediately after the surgery, animals received a single injection of benzylpenicillin benzathine (7 mg/kg i.m.). For mean arterial pressure (MAP) and heart rate (HR) recordings, the catheter was connected to a pressure transducer (Cobe Laboratories), which was plugged to a pressure-processor amplifier and data acquisition system (MP100; Biopac Systems). MAP and HR direct recordings were obtained 48 h after the catheter placement. During this procedure, the animals were conscious and moving freely in their cages.

Isolation of BM cells

BM cells were obtained from femurs and tibias of mice euthanized with a sodium thiopental overdose (100 mg/kg, i.p.). After cleaning all soft tissue, epiphyses were removed to gain access to the marrow cavities and whole BM was flushed out with the Dulbecco's modified Eagle's medium (Sigma-Aldrich). Then, BM cell suspension was incubated twice with a lysing buffer 1 $\times$ (BD) for 5 min at 37°C to remove erythrocytes. The cell suspension was subsequently centrifuged for 10 min at 1200 rpm, the supernatant was discarded, the pellet was washed and resuspended in phosphate-buffered saline (PBS; Sigma-Aldrich) plus 1% fetal bovine serum (Sigma-Aldrich).

Measurement of intracellular ROS

The ROS analysis was performed by flow cytometry as previously described (Campagnaro *et al.*, 2013; Tonini *et al.*, 2013). Dihydroethidium (DHE) and 2',7'-dichlorofluorescein diacetate (DCF-DA) were used to detect intracellular $\bullet\text{O}_2^-$ and H_2O_2 , respectively. By its ability to freely permeate cell membranes, DHE is extensively used to monitor $\bullet\text{O}_2^-$ production. Upon reaction with $\bullet\text{O}_2^-$, DHE is rapidly oxidized forming ethidium, a red fluorescent product that intercalates with DNA and amplifies the red fluorescence signal. DCF-DA is a cell permeant indicator for H_2O_2 production that is

nonfluorescent until oxidation occurs within the cell, converting it to the fluorescent form and which remains trapped in the cell. DHE (160 μM) and DCF-DA (20 mM) were added to cell suspension (10^6 cells) and incubated at 37°C for 30 min, in the dark, to estimate intracellular $\bullet\text{O}_2^-$ or H_2O_2 concentration (Tonini *et al.*, 2013). For positive control, samples were treated for 5 min with 50 μM H_2O_2 to create an oxidative stress without being toxic to the cells, whereas for negative control, the cells were incubated with ethanol. Cells were then washed, resuspended in PBS, and kept on ice for an immediate detection by flow cytometry (FACSCanto II, Becton Dickinson, San Juan, CA). Data were analyzed using the FACSDiva software (Becton Dickinson) and overlay histograms were constructed using FCS Express software trial (De Novo). For quantification of DHE and DCF fluorescence, samples were acquired in triplicate and 10,000 events were used for each measurement. Cells were excited at 488 nm and DHE and DCF fluorescence were detected using, respectively, 585/42 and 530/30 bandpass filters and data expressed as the median fluorescence intensity (MFI).

DNA content analysis

Cell cycle distribution was determined by flow cytometry analysis for the DNA content. Briefly, 10^6 BM cells were fixed in cold 70% ethanol for 2 h at -20°C . Cell samples were washed, resuspended in ice-cold PBS, and incubated with 200 μL of staining solution (20 mg/mL RNase A, 500 $\mu\text{g}/\text{mL}$ propidium iodide [PI], 1% Triton X-100) for 30 min at 4°C , in the dark. Then, cells were washed, centrifuged for 10 min at 1200 rpm, and resuspended in PBS. Samples were acquired in triplicate and 10,000 events were used for each measurement. The cell cycle profile was determined using a FACSCanto II flow cytometer. For determination of DNA content, samples were acquired in triplicate and 10,000 events were used for each measurement. The PI method for detecting fragmented DNA relies on the principle that the marker enters the cell after membrane permeabilization labeling DNA. In addition, the fragmented low-molecular weight DNA can be observed to the left of interphasic DNA (2n) cells peak (Yen *et al.*, 2010). Data analysis was performed by the FACSDiva software via observation of the following: the percentage of fragmented DNA, the interphasic DNA, which indicates that the cells are not dividing, and duplicated DNA, which indicates that the cells are undergoing mitosis.

Annexin V and PI assay

Apoptotic cells were determined by the loss of plasma membrane integrity, which was characterized by the translocation of the phospholipid phosphatidylserine from the inner to the outer leaflet allowing the binding of the annexin V protein to cells with exposed phosphatidylserine. After isolation, 10^6 BM cells were centrifuged for 10 min at 1200 rpm, and then resuspended in 400 μL of the binding buffer 1 $\times$ (10 mmol HEPES/NaOH, pH 7.4, 140 mmol NaCl, 25 mmol CaCl_2). Then, $\sim 10^5$ cells (100 μL) were transferred to a new tube and incubated with 5 μL of Annexin V-FITC and 5 μL of PI for 15 min at room temperature (25°C) in the dark, using a commercial kit according to the manufacturer's instructions (Becton Dickinson). Finally, 400 μL of 1 $\times$ binding buffer was added to each tube and the cells analyzed by a FACSCanto II flow cytometer (Becton Dickinson) within 1 h

of staining. All the data analyses were performed using FACSDiva analysis software (Becton Dickinson). For quantification of apoptotic cells, samples were acquired in triplicate and 10,000 events were used for each measurement. Cells were excited at 488 nm and FITC and PI fluorescence were detected using, respectively, 530/30 and 585/42 bandpass filters and data expressed as the percentage of positive cells. The percentage of cells was determined in Q_1 (Annexin V^{FITC}⁻/PI⁺, damaged cells), Q_2 (Annexin V^{FITC}⁺/PI⁺, end stage apoptotic and death cells), Q_3 (Annexin V^{FITC}⁻/PI⁻, viable cells), and Q_4 (Annexin V^{FITC}⁺/PI⁻, early apoptotic cells) (Tonini *et al.*, 2013). The apoptotic rate of cells undergoing apoptosis was determined as the percentage of Q_2+Q_4 .

Statistical analysis

Data are presented as either representative figures or as mean $\pm$ SEM. Flow cytometry data of ROS production are expressed as MFI $\pm$ variation coefficient of three repeated and statistically reproducible (the Friedman test) measurements of at least five independent animals. The normality of the variables was evaluated using the Kolmogorov–Smirnov test. When this test was significant, the statistical analysis was performed using the Student's *t*-test for comparison of two independent groups. When the test of normality was not

significant, the statistical analysis was performed using the nonparametric Mann–Whitney test. The differences between means with a value of $p < 0.05$ were considered statistically significant.

Results

Blood pressure and HR

Figure 1 shows average values of direct resting MAP and HR measurements in conscious animals 14 days after renal artery clipping. As expected, 2K1C mice showed higher MAP than sham mice (144 ± 6 vs. 103 ± 1 mmHg, $p < 0.01$), which was accompanied by tachycardia when compared with sham mice (612 ± 38 vs. 483 ± 20 bpm, $p < 0.05$), confirming 2K1C hypertension development and establishment.

ROS production

We evaluated the production of ROS using flow cytometry with DHE and DCF-DA to quantify the production of $\bullet O_2^-$ (Fig. 2) and H_2O_2 (Fig. 3), respectively; the presence of both compounds was indicated by the MFI (in a.u.). Typical histograms from flow cytometric analysis show a rightward shift in the log of DHE fluorescence in 2K1C mice compared with sham mice (Fig. 2A). As summarized in Figure 2B, we

FIG. 1. Hemodynamic features. Direct mean arterial pressure and heart rate measurements 2 weeks after clip placement or sham operation in conscious hypertensive (two-kidney, one-clip [2K1C]) and normotensive (sham) mice. The values are presented as mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ versus the sham group (the Student's *t*-test for independent samples).

FIG. 2. Production of superoxide anions. Representative histograms from flow cytometric analysis using dihydroethidium (DHE) in sham compared with 2K1C mice (A); the log X axis illustrates the intensity of fluorescence for the number of cells counted. A remarkable increase in the level of superoxide anions was observed in hypertensive mice. (B) Bar graph shows median fluorescence intensity (MFI). The data shown are representative of three independent and statistically reproducible experiments (the Friedman test). The values are presented as mean $\pm$ SEM. * $p < 0.05$ versus the sham group (Mann–Whitney test).

FIG. 3. Production of hydrogen peroxide (H_2O_2). Representative histogram from flow cytometric analysis with 2',7'-dichlorofluorescein diacetate (DCF-DA) comparing a sham with a 2K1C mouse (**A**); the log X-axis illustrates the intensity of fluorescence for the number of cells counted. Increased levels of H_2O_2 were observed in 2K1C hypertensive mice. (**B**) Bar graph shows MFI. The data shown are representative of three independent and statistically reproducible (the Friedman test) experiments. The values are presented as median $\pm$ SEM. * $p < 0.05$ versus the sham group (the Mann-Whitney test).

observed a remarkable increase in the levels of $\bullet\text{O}_2^-$ in hypertensive mice compared with normotensive mice (sham: 2058 ± 891 vs. 2K1C: 11971 ± 2861 a.u., $p < 0.05$). Similarly, the production of H_2O_2 was significantly increased in the 2K1C group compared with the sham group (sham: 4007 ± 653 vs. 2K1C: 7935 ± 1437 a.u., $p < 0.05$). Typical histograms from flow cytometric analysis with DCF-DA are shown in Figure 3A and summarized in Figure 3B.

DNA content

Given that 2K1C hypertension increases ROS production, and that this stimulus can interact with DNA, we tested the cell cycle distribution and DNA fragmentation by cell permeabilization followed by PI labeling. The genetic content of BM cells was evaluated by flow cytometry via the observation of the percentage of fragmented, interphasic, and duplicated DNA. As illustrated in the DNA histograms (Fig. 4), 2K1C (Fig. 4B) hypertension showed a subtle increase in fragmented DNA on BM cells compared to sham (Fig. 4A) mice. The total fragmented DNA is summarized in Figure 4C, confirming the augmented DNA fragmentation in hypertensive mice (sham: $6.51\% \pm 0.4\%$ vs. 2K1C: $11.95\% \pm 0.8\%$, $p < 0.05$). On the other hand, no significant difference was observed for interphasic and duplicated DNA.

Apoptotic cell rate

Considering that during early phases of apoptosis, phosphatidylserine, a protein usually located in the inner leaflet of the plasma membrane, translocates to the outer layer, becoming available for Annexin V binding and that PI is a nucleic acid-specific marker that is excluded from live cells, but stains DNA and RNA once the plasma membrane is disrupted, we evaluated the rate of apoptosis in BM cells through flow cytometry using Annexin V^{FITC} and PI to distinguish live and healthy cells (Q3: Annexin V^{FITC}⁻/PI⁻) from early apoptotic cells (Q4: Annexin V^{FITC}⁺/PI⁻) and late apoptotic or necrotic cells (Q2: Annexin V^{FITC}⁺/PI⁺). Figure 5A shows representative dot-plots for 2K1C and sham groups showing a remarkable increase in apoptotic cell number (Q2+Q4) in hypertensive compared with sham mice. As demonstrated in Figure 5B, 2K1C hypertension significantly increased the percentage of BM apoptotic cells (sham: $14.4\% \pm 3.7\%$ vs. 2K1C: $34.9\% \pm 7.2\%$, $p < 0.05$) compared to sham mice.

Discussion

Oxidative stress

The findings obtained by this study corroborate our previous reports (Campagnaro *et al.*, 2012, 2013) supporting the hypothesis that 2K1C hypertension might induce oxidative stress in nonspecific tissues, including the BM. Enhanced levels of oxygen radicals, which characterize a state of oxidative stress, have been described in clinical and preclinical studies in many cardiovascular diseases, including diabetes, hypertension, and atherosclerosis (Minuz *et al.*, 2002; Porto *et al.*, 2011; Silva *et al.*, 2012; Vasquez *et al.*, 2012; Luévano-Contreras *et al.*, 2013). Indeed, an excess of either molecular oxygen or chemical derivatives of oxygen (Kodja and Harrison, 1999) may be a common underlying pathogenic mechanism in these diseases. Among ROS, attention has focused on the highly reactive free radical $\bullet\text{O}_2^-$ and the more stable H_2O_2 (Touyz and Schiffrin, 2004). The $\bullet\text{O}_2^-$ is considered the precursor of all ROS because it is the first produced. On the other hand, H_2O_2 is a highly stable ROS generated mainly by $\bullet\text{O}_2^-$ dismutation. Despite different sources of ROS, there is consensus that the NADPH oxidases are the main generators of ROS in the vasculature during diseases (Touyz, 2000; Viridis *et al.*, 2011). Compelling data implicate ROS generated by NADPH oxidases in the pathophysiology of renovascular hypertension (Salguero *et al.*, 2008; Endtmann *et al.*, 2011).

Considering that the BM is a highly organized and complex organ system that gives rise to all mature and circulating blood cells, and that BM has been shown to carry a complete RAS (Strawn *et al.*, 2004), it is reasonable to suggest that local Ang II can be responsible for ROS production in the BM of 2K1C hypertensive mice. However, given the ubiquitous nature of the RAS, separating the effects of circulating and localized Ang II is quite difficult.

In conditions of RAS overactivity, such as arterial hypertension (Touyz, 2000), ROS generation becomes a significant contributor to cellular oxidation, since Ang II can induce H_2O_2 and $\bullet\text{O}_2^-$ formation via activation of NADPH oxidase (Paravicini and Touyz, 2006; de Cavanagh *et al.*, 2007). Interestingly, *in vitro* studies showed that the NADPH oxidase

FIG. 4. Flow cytometric detection of apoptosis with Annexin V^{FITC} and PI. **(A)** Representative dot-plots of Annexin V^{FITC} and PI dual color flow cytometry for BM cells of sham and 2K1C mice are shown. Top left quadrant (Q1) shows damaged cells (Annexin V^{FITC}⁻/PI⁺). Top right quadrant (Q2) contains cells in late apoptosis or already dead (Annexin V^{FITC}⁺/PI⁺). Bottom left quadrant (Q3) shows viable cells (Annexin V^{FITC}⁻/PI⁻). Bottom right quadrant (Q4) represents cells that are in early apoptosis (Annexin V^{FITC}⁺/PI⁻). Note a remarkable increase in apoptotic cell number (Q2+Q4) in the 2K1C animals. **(B)** Bar graph shows average percentage of apoptotic cells (Q2+Q4). The data shown are from three independent and statistically reproducible (the Friedman test) experiments. Values are mean $\pm$ SEM. * $p < 0.05$ versus the sham group (the Student's *t*-test for independent samples). BM, bone marrow.

activity is regulated by both Ang II (Hayakawa *et al.*, 1997; Haynes *et al.*, 1998) and shear stress (Haynes *et al.*, 1997). However, it is not clear whether *in vivo* Ang II or cyclical stretch or both are involved in the augmented oxidative stress observed in the 2K1C mice. In a hypertension rat model of infused Ang II, was demonstrated that this peptide, but not blood pressure is responsible for ROS production (Cubeddu *et al.*, 2000).

It should be taken into account that in cardiovascular diseases, such as hypertension, increased oxidative stress is directly correlated with cardiac and vascular sympathetic tone overactivity (Abboud, 2010; Campos *et al.*, 2011). In the previous study, we observed an imbalance of the autonomic control of the cardiovascular system (sympathetic overactivity and impaired parasympathetic activity) in the 2K1C model of renovascular hypertension (Peotta *et al.*, 2007). Moreover, augmented ROS production mediates the Ang II actions and results in a sympathetic tone imbalance (Zimmerman *et al.*, 2004; Gao *et al.*, 2005; Oliveira-Sales *et al.*, 2009), which worsens cardiovascular disease processes (Abboud, 2010; Campos *et al.*, 2011). Additionally, in models of infused or endogenous (2K1C) Ang II, inhibition of ROS partly attenuates hypertension, indicating that Ang II-induced ROS is important for vasoconstriction in these models (Taniyama and Griendling, 2003). We speculate that the sympathetic overactivity that accompanies the renovascular hypertension in this model could be an additional mechanism that leads to or contributes to the BM apoptosis observed in renovascular hypertension.

Despite the impact of arterial hypertension on ROS production in BM cells is poorly understood in murine models,

our data support the idea that 2K1C hypertension contributes to oxidative stress in BM cells, leading to unfavorable processes, such as DNA damage (Schupp *et al.*, 2007; Campagnaro *et al.*, 2013) and apoptosis (Endtmann *et al.*, 2011).

DNA content

Maintenance of genomic integrity is essential to survival. However, exogenous agents and/or endogenous important biological processes generate ROS, which in elevated levels are able to cause damage to macromolecules, such as nucleic acids, proteins, and lipids (Evans *et al.*, 2004). To combat the harmful effects of ROS, living cells have acquired a number of defenses (Evans *et al.*, 2004). However, under pathological conditions, when ROS levels are dramatically increased, these repair systems do not mitigate all damages leading to DNA fragmentation.

Some authors, including us, have observed alterations in DNA caused by hypertension (Schupp *et al.*, 2007; Fandos *et al.*, 2009; Campagnaro *et al.*, 2012, 2013). In this work, the DNA content analysis showed that 2K1C hypertension induces DNA fragmentation in BM cells, suggesting that the disease leads to DNA changes compromising the functionality of the cells. Moreover, others have shown that Ang II-induced hypertension leads to DNA damage in mouse kidneys (Fazeli *et al.*, 2012; Brand *et al.*, 2013) and heart (Brand *et al.*, 2013), and that these genotoxic effects were mediated by ROS, once tempol treatment was able to inhibit DNA damage (Brand *et al.*, 2013).

A series of remarkable studies suggest that injury to a target organ is sensed by distant stem cells, mostly localized

FIG. 5. DNA content analysis. Permeabilized bone marrow cells were labeled with propidium iodide (PI) and DNA content evaluated by flow cytometry. Typical histograms of cell cycle distribution from sham (A) and 2K1C (B) mice. (C) Bar graph shows percentage of cells with fragmented DNA. The data shown are from three independent and statistically reproducible (the Friedman test) experiments. Values are mean $\pm$ SEM. * $p < 0.05$ versus the sham group (the Student's t -test for independent samples).

in BM tissue, which migrate to promote structural and functional repair. Under physiological conditions, the generation of ROS is relatively lower in BM-derived stem cells compared to cycling cells, which potentially could reduce the frequency of DNA damage (Arai *et al.*, 2004; Kiel *et al.*, 2007; Yoshihara *et al.*, 2007). However, in pathophysiological states, such as hypertension, cell differentiation and proliferation may increase the level of ROS in BM causing DNA damage (Sattler *et al.*, 1999; Milovanova *et al.*, 2009; Tesio *et al.*, 2011). Accordingly, we recently demonstrated that intracellular-generated ROS can lead to DNA damage in mononuclear cells of 2K1C hypertensive mice (Campagnaro *et al.*, 2013). Thus, the accumulation of DNA fragmentation in BM cells, reflecting defects in the repair machinery, which in turn indicates compromised genome maintenance. Therefore, we hypothesize that when genomic lesions are extremely extensive, cellular apoptosis may occur and diminish

cell viability and integrity, which could be a disadvantage to the use of this cell source for transplantation in human beings.

Apoptosis

Apoptosis is the most well-defined morphological and biochemical type of cell death pathway. Oxidative stress has been shown to be one of the key proapoptotic factors since it directly damages cell membranes, proteins, and DNA; thereby, promoting cell senescence, comprising cell function and threatening cell survival (Wei *et al.*, 2010; Zanichelli *et al.*, 2012). ROS are involved in many of the physiological and pathological processes observed in hypertension, since it is capable of inducing oxidation and damage of macromolecules contributing to cellular damage and, consequently, cell death through apoptosis (Lavi *et al.*, 2010; Endtmann *et al.*, 2011; Worou *et al.*, 2011). These processes have been documented in different cell systems (Rodriguez-Lopez *et al.*, 1998; Rizzoni *et al.*, 2000; Liu *et al.*, 2003), but apoptosis in the 2K1C hypertension model is not completely understood. In the present study, we examined the hypothesis that DNA fragmentation and apoptosis are consequences of ROS production and determined whether these events also occur in BM cells.

ROS such as $\bullet\text{O}_2^-$ and H_2O_2 , which we found augmented in 2K1C hypertensive mice play an important role in normal cell growth, migration, differentiation, apoptosis, and senescence (Griendling *et al.*, 2000; Finkel, 2003). Our data corroborate the finding that renal artery clipping leads to augmentation of Ang II and ROS production (Lerman *et al.*, 2001), which are well known to triggers the apoptotic process (Elahi *et al.*, 2008). Others have focused on the intracellular mechanisms responsible for Ang II-induced apoptosis (Paravicini and Touys, 2006). It has been shown that direct cellular responses to proliferation, differentiation, mitosis, and apoptosis involve the mitogen-activated protein kinase (MAPK) family (Lee *et al.*, 1994). Accordingly, Endtmann *et al.* (2011) demonstrated that Ang II, acting through the AT1 receptor, activates proapoptotic signaling pathways in endothelial progenitor cells, by enhancing phosphorylation of ASK-1, *c-Jun* N-terminal kinase, and p38^{MAPK} . The activation of those pathways leads to decreased expression of anti-apoptotic *Bcl-2*, and increased expression of proapoptotic *Bax* (Endtmann *et al.*, 2011). There is evidence that p38^{MAPK} , a protein that typically mediates apoptosis and inflammatory responses, is markedly involved in injured cardiovascular cells by a variety of stimuli, including Ang II and ROS (Zhang *et al.*, 2009; Endtmann *et al.*, 2011). Augmented production of H_2O_2 , as observed in the present study, is known to activate the apoptosis marker p38^{MAPK} (Wei *et al.*, 2010). In addition, several *in vitro* studies suggest that Ang II-induced activation of NADPH oxidase results in upregulation of p38^{MAPK} (Lal *et al.*, 1999; Chan *et al.*, 2005). A recent study demonstrated that Ang II induces apoptosis in a mitochondria-dependent manner activated by ROS, which triggers the release of cytochrome *c* from mitochondria and also decreases the expression of the antiapoptotic protein *Bcl-2*, leading to the activation of caspase-9 and -3, thus initiating apoptosis (Chen *et al.*, 2011). Oxidative DNA damage induces apoptosis through genomic or nongenomic mechanisms resulting in mitochondrial DNA damage and

compromising the energy supply and consequently apoptosis (Polyak *et al.*, 1997; Mercer *et al.*, 2007; Yu *et al.*, 2012).

Recently, Endtmann *et al.* (2011) showed that Ang II was able to induce endothelial progenitor cell apoptosis *in vitro* via the AT1 receptor. The present study corroborates previous observations that $\bullet\text{O}_2^-$ and H_2O_2 in excess amounts limits the self-renewing capacity and life span of BM hematopoietic stem cells, resulting in apoptosis (Ito *et al.*, 2006; Wang *et al.*, 2011). The importance of the present study is that the Annexin V^{FITC}/PI assay allowed us to demonstrate an increased apoptosis rate of BM cells in renovascular hypertensive animals.

Considering the increasing number of studies describing BM cell transplantation as a strategy for cardiovascular repair, the therapeutic efficacy of this procedure could be greatly limited by augmented apoptosis. Thus, this study suggests that comorbidities should be particularly considered if autologous transplantation is intended, since Ang II-dependent hypertension compromises the integrity, viability, and, consequently, functionally of the BM to produce the stem/progenitor cells responsible for end-organ structure and function repair.

Conclusion

The novelty of this study is the finding that 2K1C renovascular hypertension leads to BM cells apoptosis, which could impair tissue repair, probably due to augmented ROS production. Further studies should be designed to determine the diminished functionality of stem/progenitor cells found in BM.

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Disclosure Statement

No competing financial interests exist.

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