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Treg cells depletion is a mechanism that drives microvascular dysfunction in mice with established hypertension

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ABSTRACT

Background: Microvascular dysfunction is a major complication in hypertensive patients. We previously reported that $CD4^+CD25^+$ T regulatory cells (Treg) play an important preventive role in hypertension-induced vascular dysfunction. However, whether Treg cells therapy and autophagy inhibition could rescue Treg cells survival and microvascular function in established hypertension is an important question that remained unanswered.

Methods & results: Here we showed that Treg cells from mice model of established hypertension displayed an enhanced apoptotic rate, which was rescued with Treg cells transfer and autophagy inhibition. We also showed increased autophagy in mesenteric resistance artery (MRA) in mice with established hypertension. Importantly, the inhibition of autophagy or one single transfer of Treg cells into mice with established hypertension improved the microvascular function independently of high blood pressure. The protection involves the modulation of interleukin-10 (IL-10), inflammation, endoplasmic reticulum (ER) stress, oxidative stress, Akt, and eNOS.

Conclusions: The present study suggests that Treg cells survival is regulated by autophagy. Also, Treg cells as a cellular therapy aimed at rescuing the microvascular function through an autophagy-dependent mechanism and independently of arterial blood pressure lowering effects. Because our mouse model of established hypertension mimics the clinical situation, our results have the potential for new therapeutic approaches that involve the manipulation of Treg cells and autophagy to overcome established hypertension-induced cardiovascular complications.

1. Introduction

It is well established that the cardiovascular system is compromised in patients with established hypertension and associated with a substantial increase in the rate of cardiovascular morbidity and mortality [1–3]. Clinical observations reported that impaired arterial function and structures predict a poor outcome in patients with cardiovascular diseases [4–6]. However, the cellular and molecular mechanisms that cause microvascular dysfunctions in established hypertension are not completely understood. Moreover, with the current medications [7–10], the incidence of established hypertension associated with cardiovascular complications continues to rise by epidemic proportions. Therefore, there is an essential need for a new therapeutic strategy to stop the progression of hypertension-dependent cardiovascular complications. In 2007, Dr. Harrison's laboratory reported that the immune T cells play an essential role in the initiation and maintenance of experimental hypertension-induced vascular dysfunction in mouse infused with angiotensin II (Ang II) [11]. Also, Dr. Muller's group showed that one single injection of the immune regulatory $CD4^+CD25^+$ (Treg) cells before the development of hypertension prevented cardiac damage in mice infused with Ang II independently of high blood pressure [12]. In 2011, we were the first to determine that the transfer of Treg cells before the development of hypertension prevented hypertension and vascular dysfunction in mice infused with Ang II [13,14]. Dr. Schiffrin's laboratory supported our studies and elucidated that Treg cells play an essential upstream role in the regulation of the cardiovascular function [15–19]. We also showed that the preventive role of Treg cells in cardiovascular dysfunction hypertension-dependent involves oxidative stress and inflammation [13,14].

Autophagy is an underlying physiological cellular process which has

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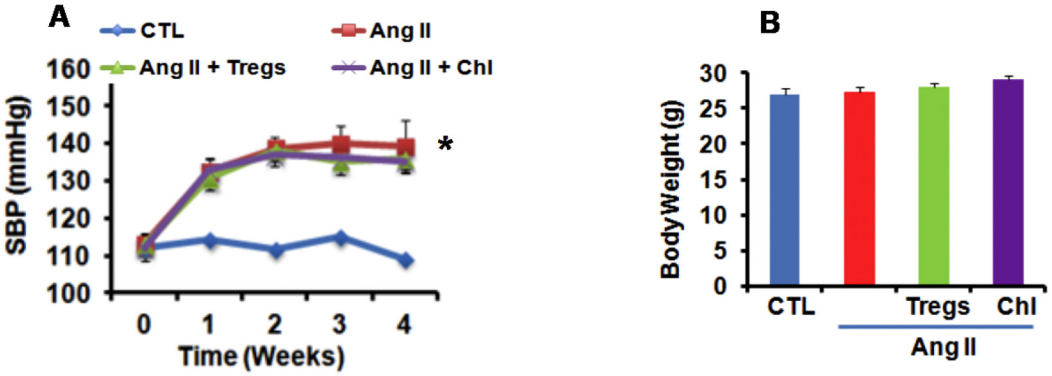


Fig. 1. Systolic arterial blood pressure (A) and body weight (B) in control (CTL), angiotensin II (Ang II), Ang II + Treg cells, and Ang II + chloroquine (Chl) mice; (n = 4–8), *p < 0.05 for Ang II vs. CTL, Ang II + Treg cells, and Ang II + Chl. Two-way repeated measure ANOVA followed by Tukey's post hoc test were applied for A, One way ANOVA followed by Tukey's post hoc test were applied for B.

a significant role in cellular homeostasis maintenance, regulation of the ER stress, removing damaged organelles and pathogens [20,21]. Whether Treg cells survival is dependent on autophagy mechanism and whether Treg cells transfer could rescue cardiovascular structure and function in a mouse with established hypertension are important questions that remained unanswered. Unfortunately, physicians often face patients with established hypertension at an advanced stage when cardiovascular complications have already occurred in most of the patients. Therefore, manipulating Treg cells population in an animal model of established hypertension is critical. This approach has the potential for clinical studies using Treg cells as a cell therapy.

The proper folding of proteins made in the endoplasmic reticulum (ER) requires oxygen for disulfide bond formation [22]. Regular ER homeostasis can be affected by different pathological factors such hypoxia and oxidative stress that affect the oxygen level leading to ER stress induction. Experimental and clinical research indicated that ER stress is involved in hypertension and cardiovascular diseases [23]. Also, one of the key cellular responses to established hypertension could be the up-regulation of autophagy [24]. Whether augmented autophagy is an essential mechanism in Treg cells apoptosis and microvascular complications under established hypertension is still unknown. These studies will advance the field forward and have the potential for novel therapies including Treg cell transfer and autophagy inhibition to rescue the microvascular system in patients with established hypertension.

2. Materials and methods

2.1. Mice

All experimental protocols and procedures follow the NIH Guide for the Care and Use of Laboratory Animals. All these procedures were approved by institutional animal care and use committee (IACUC) at Eastern Virginia Medical School, VA, USA.

Wild types (C57BL/6J) 8 weeks-old males were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained at the EVMS animal care facility under pathogen-free conditions, housed at a temperature of 23 °C with 12 h light/dark cycles and had unrestricted access to food and water.

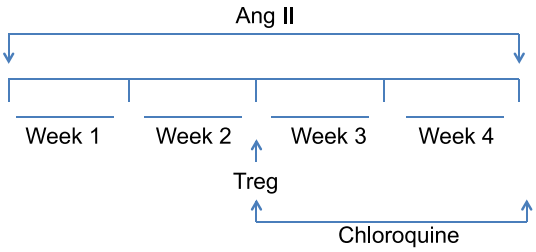
Mice were divided into 4 groups:

Group 1: Wild type mice (C57BL/6 J, CTL, n = 8) as a control without treatment.

Group 2: Wild type mice (C57BL/6 J) infused with Ang II (400 ng/kg/min) for 4 weeks (Ang II, n = 8).

Group 3: Mice infused with Ang II for 4 weeks. These mice received one single dose of Treg cells (200,000 cells, ip) at the beginning of the third week (Ang II + Tregs, n = 8). Treg cells were isolated from spleens of control mice.

Group 4: Mice infused with Ang II for 4 weeks. These mice were injected with autophagy inhibitor (chloroquine "Chl", 10 mg/kg/day, ip) at the beginning of the third week for 2 weeks (Ang II + Chl, n = 8).



The infusion of Ang II was performed using subcutaneous mini-osmotic pumps (Alzet-Model 1004; Durect Corporation, Cupertino, CA) and systolic blood pressure was recorded weekly using the CODA tail-cuff blood pressure system (Kent Scientific Torrington, USA) [14,25]. Blood pressure measurements were performed at the same time of the day (between 9 am and 11 am) in order to avoid the influence of the circadian cycle, and the value of SBP was obtained by estimating the average of 6–8 measurements. At the end of the treatment period, mice were sacrificed and mesentric resistance areries (MRA) were harvested immediately. Blood samples were obtained immediatley and centrifuged at 2500 rpm for 10 min at 4 °C to obtain plasma, which was immediately stored at −80 °C.

2.2. Treg cells isolation and transfer

Treg cells were isolated from control mice spleen using $CD4^+CD25^+$ Regulatory T Cell Isolation Kit mouse, Miltenyi Biotech MACS as previously reported [13,14] and then injected into hypertensive mice at the beginning of third week.

2.3. Vascular reactivity

Vascular reactivity was performed as reported previously [13,14,25] in MRA isolated from control (CTL), Ang II, Ang II + Treg cells, and Ang II + Chloroquine (Chl) groups of mice. The MRAs were immediately placed in PSS solution (NaCl 118 mM; KCl 4.7 mM; $CaCl_2$ 2.5 mM; KH_2PO_4 1.2 mM; $MgSO_4x7H_2O$ 1.2 mM; $NaHCO_3$ 25 mM and glucose 11 mM, pH = 7.4). MRA were then cleaned of fat and connective tissue and then cut into rings (2 mm in length). Then rings were mounted in a small vessel dual chamber myograph for measurement of isometric tension. After a 30 min equilibration period in PSS solution

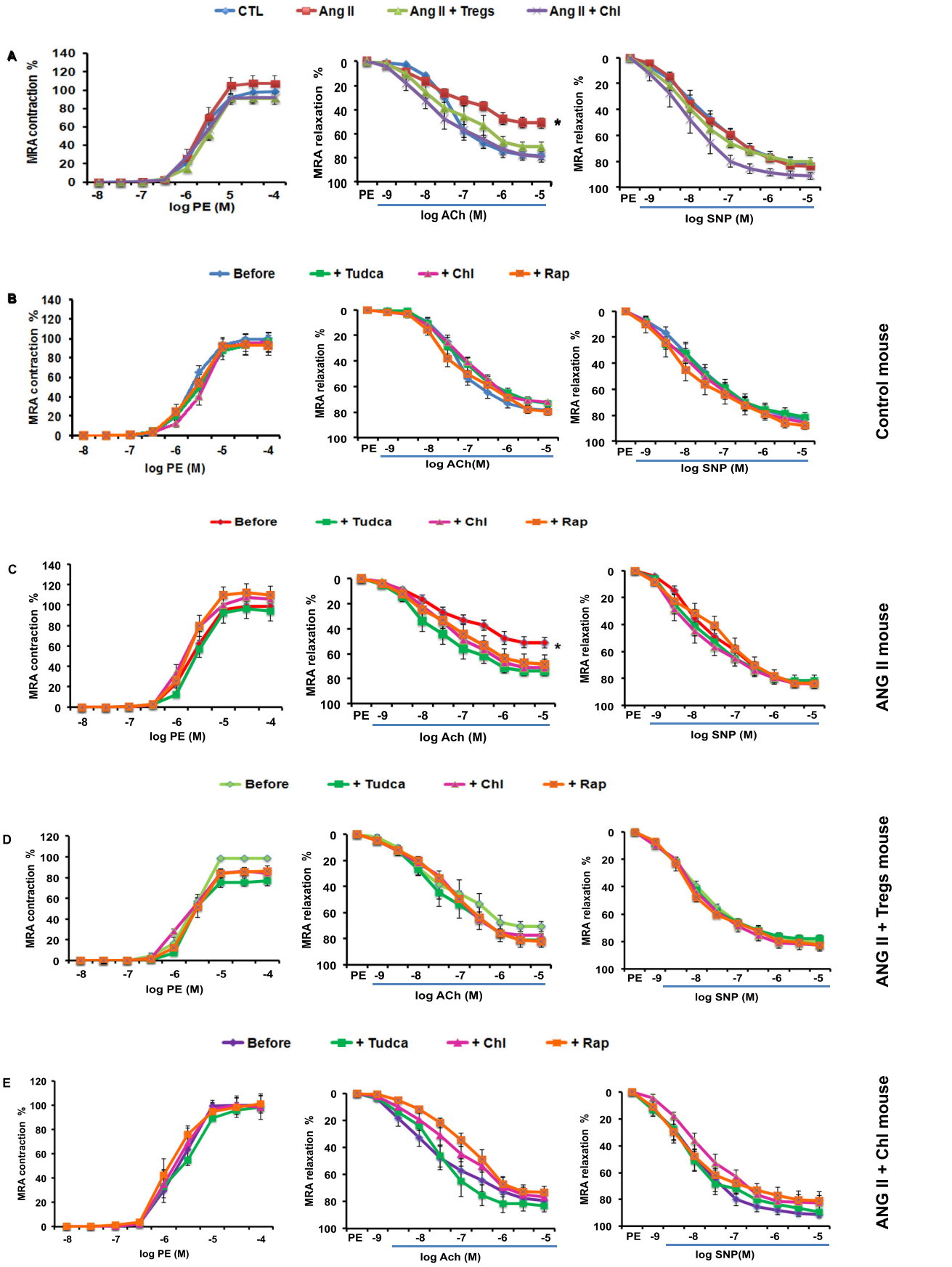


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Fig. 2. Mesenteric resistance arteries (MRA) reactivity (Wire Myograph) showing contractility in response to sympathetic stimulation (phenylephrine, PE), endothelium-dependent and independent relaxation in response to acetylcholine (ACh) and sodium nitroprusside (SNP) in control (CTL), angiotensin II (Ang II), Ang II + Treg cells, and Ang II + Chloroquine (Chl) mice (A). MRA reactivity in CTL, Ang II, Ang II + Treg cells, and Ang II + Chl incubated with and without ER stress inhibitor (Tudca), autophagy inhibitor (Chl), and mTOR signaling inhibitor (Rapamycin, Rap) (B, C, D, E), (n = 5–6), *p < 0.05 for Ang II vs. CTL, Ang II + Treg cells, and Ang II + Chl. Two-way repeated measure ANOVA followed by Tukey's post hoc test were applied for A–E.

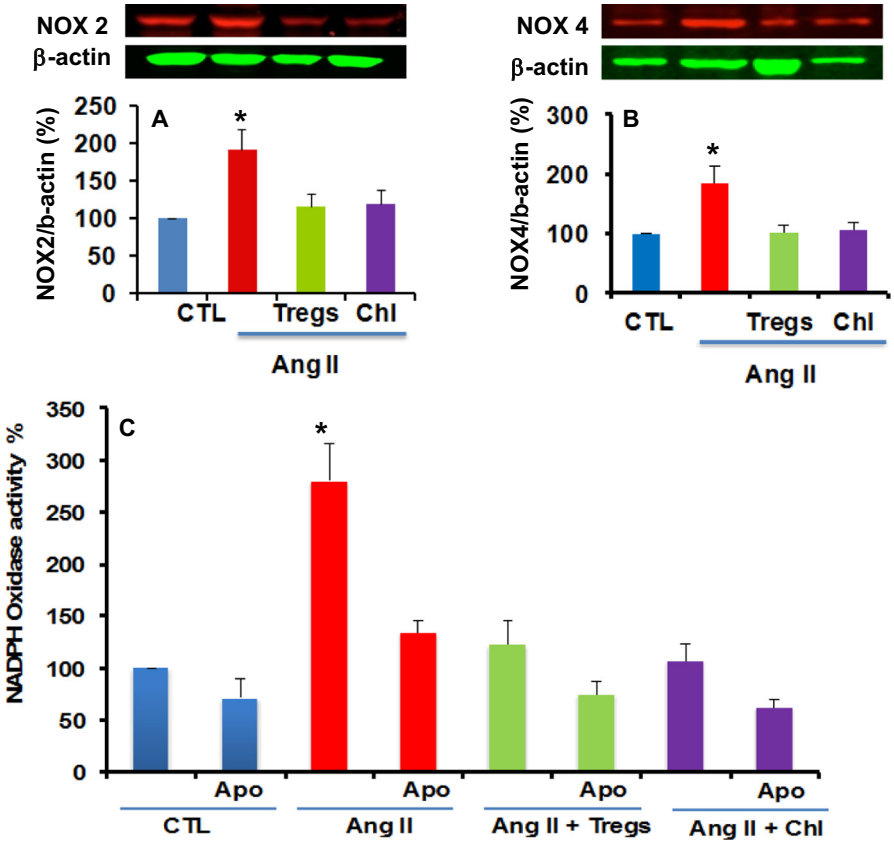

Fig. 3. Oxidative stress assessed by Nox2, Nox4 expression and NADPH oxidase activity with and without incubation with Apocynin (APO) (A, B, C) in mesenteric resistance arteries from control (CTL), angiotensin II (Ang II), Ang II + Treg cells, and Ang II + chloroquine (Chl) mice; (n = 4–6), *p < 0.05 for Ang II vs. CTL, Ang II + Treg cells, and Ang II + Chl. One way ANOVA followed by Tukey's post hoc test were applied for A–C.

bubbled with carbogen at 37 °C and pH = 7.4, arteries were stretched to their optimal lumen diameter for active tension development. After one-hour incubation, cumulative concentration responses to phenylephrine (PE, 10^{-8}–10^{-4} M) were obtained. A plateau was obtained before moving from one dose to another dose. After washing, rings were pre-constricted with PE (10^{-5} M) and when a steady maximal contraction was reached, cumulative concentration-response curves were obtained for acetylcholine (ACh, 10^{-9}–10^{-5} M) and sodium nitroprusside (SNP, 10^{-9}–10^{-5} M).

To determine the role of autophagy, ER stress and mTOR pathway in vitro, we performed experiments where we isolated MRA from each group and incubated with ER stress (Tudca), autophagy (Chloroquine) and mTOR (rapamycin) inhibitors and then cumulative concentration responses to phenylephrine (PE, 10^{-8}–10^{-4} M), acetylcholine (ACh, 10^{-9}–10^{-5} M) and sodium nitroprusside (SNP, 10^{-9}–10^{-5} M) were performed.

2.4. Western blot analysis

Western Blot was used to detect specific proteins in lysates of mice MRA tissue as previously described. [13,14,25] Mice were sacrificed then MRA were immediately harvested and frozen in liquid nitrogen and then stored at −80 °C. Tissue lysates were prepared by homogenizing with electrical homogenizer after adding ice cooled lysis buffer (T-PER Tissue protein Extraction Reagent (Prod# 78510 Thermo scientific), sonicated for 5 s, and centrifuged for 15 min at 13,000 rpm. Protein quantification was performed according to Pierce™ BCA Protein Assay Kit (Product No. 23225).

We used specific antibodies against Anti-Phospho-Akt (ser473, Cell Signaling, #9271), Anti-total-Akt (Cell Signaling, #9272), Anti-Bip (C50B12, Cell Signaling, #3177), Anti-CHOP (Cell Signaling, #2895), Anti-Phospho-AMPKa (Thr172, Cell Signaling, #2351), Anti-AMPKα (site, Cell Signaling, #2352), Anti-Phospho-mTOR (Ser2448, Cell Signaling, #2971), Anti-Phospho-eNOS (Ser1177, Cell Signaling, #9571), Anti-Beclin1 (ab62557), Anti-Atg7 (#ab53255), Anti-Atg5 (ab108327), Anti-NOX2/gp91phox (#ab80508), Anti-FOXP3 (#ab75763), Anti-NADPH oxidase 4 (#ab133303), Anti-ATF-6α (#sc-22799), Anti-eNOS/NOS Type III (BD biosciences, #610296). All dilutions were prepared according to manifacturer. Membrane was developed using odyssey-imaging system LICOR and band quantification was performed using image J software.

2.5. Immunohistochemistry

The immunostaining was performed as previously reported [13,14,25] MRA tissues were fixed in 4% of zinc-saturated formalin and paraffin-embedding for immunoperoxidase staining using the Vectastain ABC Kit. The MRA sections were incubated overnight with the Anti-CD68 antibody (Bio-Rad, MCA1957), Anti-Myeloperoxidase (MPO) antibody (#ab9535) and Anti-FOXP3 (#ab54501). Dilutions were prepared according to the manufacturer. Sections were examined in bright field of Olympus OPELCO (BX41) Light/Fluorescence microscope.

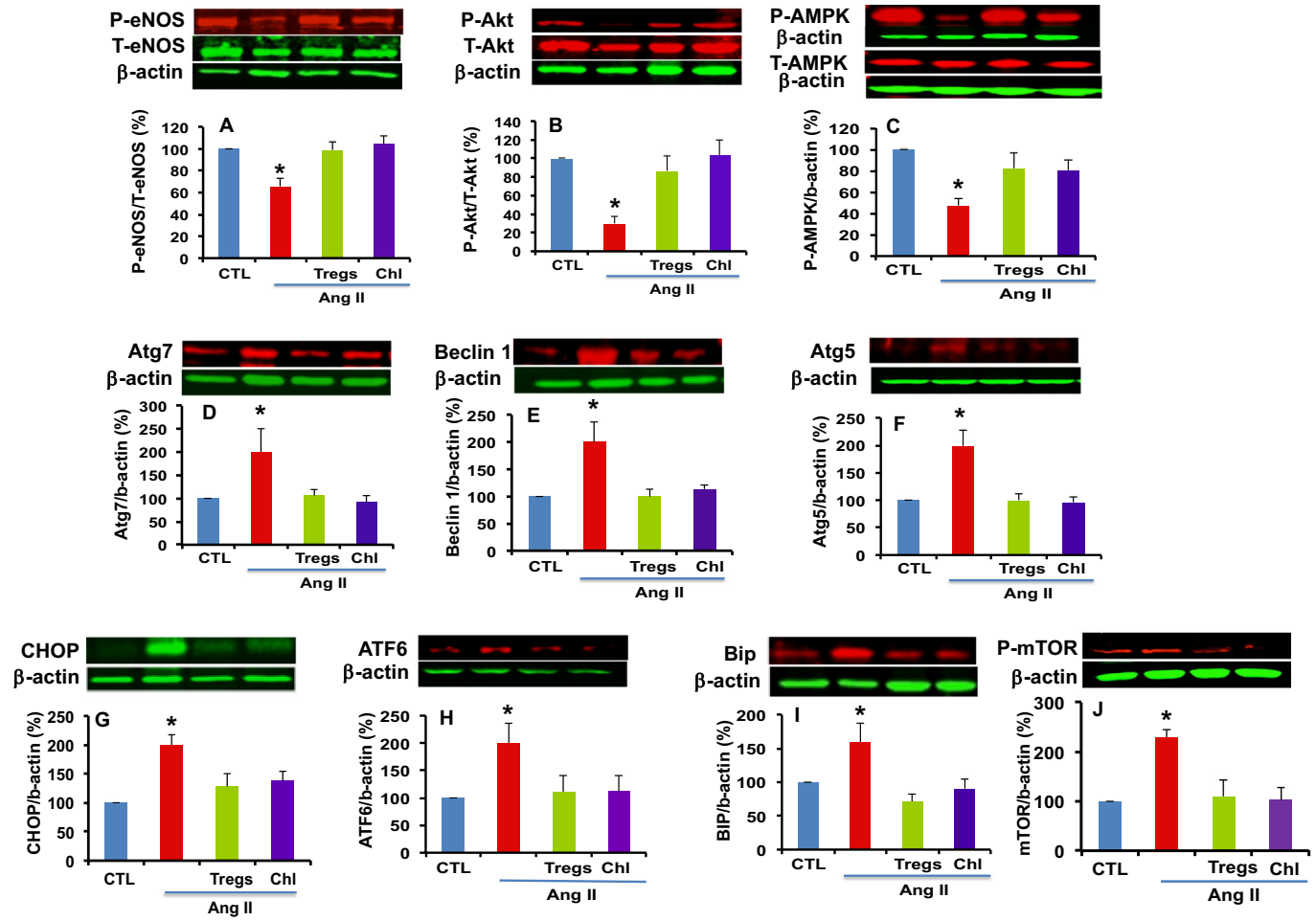

Fig. 4. Western blot analysis for markers of survival (phosphorylated and total eNOS and Akt), energy sensor (phosphorylated and total AMPK) (A, B, C), autophagy (Atg5, Beclin1, Atg7) (D, E, F), ER stress (CHOP, ATF6, BIP) (G, H, I), and mTOR (J) in mesenteric resistance arteries from control (CTL), angiotensin II (Ang II), Ang II + Treg cells, and Ang II + chloroquine (Chl) mice, (n = 4–6), *$p < 0.05$ for Ang II vs. CTL, Ang II + Treg cells, and Ang II + Chl. One-way ANOVA followed by Tukey's post hoc test were applied for A–J.

2.6. Nadph oxidase activity assay

Superoxide anion levels generated by NADPH oxidase activity were measured in lysates of MRA using lucigenin chemiluminescence as previously described [14,25]. Lysates were prepared in a sucrose buffer containing (KH_2PO_4 50 mM, EGTA 1 mM, sucrose 150 mM; pH = 7.0 and the "Complete-C mini" protease inhibitor cocktail (Roche Diagnostics, IN) in a Tissue Dounce Homogenizer on the ice. The aliquots of the homogenates were used immediately. A volume of 100 µL of each lysate was used in a total volume of 1 mL PBS buffer preheated at 37 °C, containing lucigenin (5 µM) and NADPH (100 µM). Blank samples were prepared using 100 µL of sucrose buffer. Lucigenin activity was measured every 30 s for 10 min in a luminometer (Turner biosystem 20/20, single tube luminometer) or till enzymatic activity reached a plateau. The NADPH oxidase activity was performed in the presence or absence (Apocynin). Data are expressed as % normalized to protein content (µg protein) compared to control.

2.7. Cytokines level measurements

Plasma levels of IL-6, IL-10, and TNF-α from Mice (CTL, CTL + AngII, Ang II + Tregs, Ang II + Chl) were measured using a Bioplex PRO™ reagent kit, Cat #171304070 M-64073437 (Mouse cytokines group 1).

2.8. Statistical analysis

Data are expressed as mean ± SEM. Concentration-response curves were analyzed using the GraphPad Prism 4.0 software (GraphPad, USA) and adjusted to a logistic equation. Statistical calculations for significant differences were performed using student *t*-test, one-way followed by Post-Hoc test or two-way ANOVA as appropriate. Comparisons are considered significant when $p < 0.05$.

3. Results

We infused mice with angiotensin (Ang) II (400 ng/Kg/min) for four weeks. At the end of the second week, mice were hypertensive (Fig. 1A). At the beginning of the third week, we performed a single transfer of Treg cells (harvested from control mice) or treated mice with an autophagy inhibitor (chloroquine, dose 10 mg/Kg) daily for two weeks. Data revealed that Ang II-treated animals that received Treg cells or chloroquine exhibited similar arterial blood pressure to mice infused with Ang II alone (Fig. 1A). The body weight did not change between all groups (Fig. 1B).

Resistance arteries contractility and endothelium-independent relaxation to sympathetic stimulation (PE) and nitric oxide donor (SNP) were similar in all groups (Fig. 2A). The endothelial dysfunction observed in mice with established hypertension was rescued with Treg cells transfer and autophagy inhibition (Fig. 2A). We also performed experiments where we isolated MRA from each group and incubated in

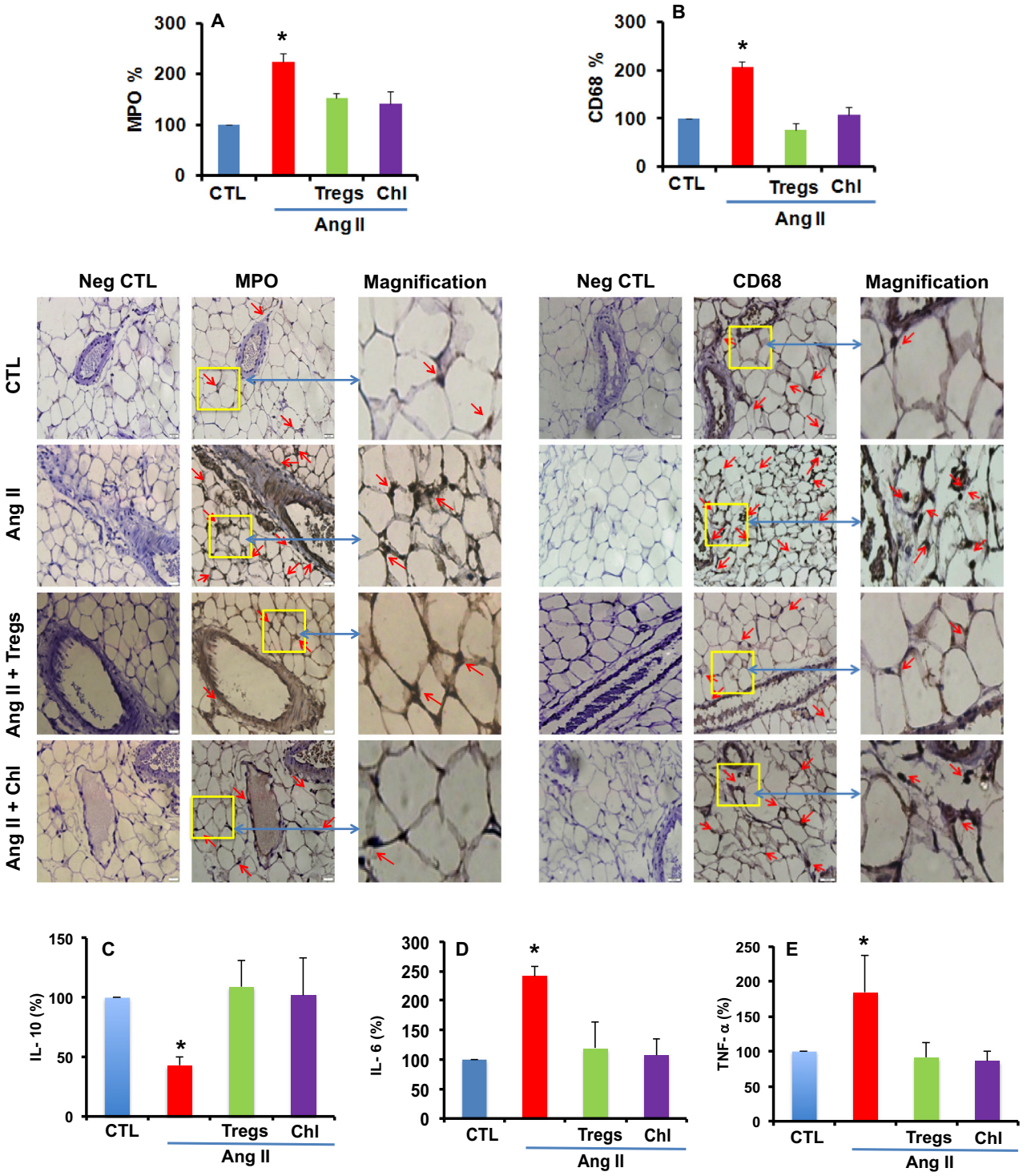

Fig. 5. Mesenteric resistance arteries inflammation assessed by the neutrophils (MPO staining) (A) and macrophages infiltration (CD68 staining) (B) into the mesenteric resistance arteries of control (CTL), angiotensin II (Ang II), Ang II + Treg cells, and Ang II + Chloroquine (Chl) mice (n = 4–5). Plasma levels of IL-10, IL-6 and TNF-α determined in control (CTL), angiotensin II (Ang II), Ang II + Treg cells, and Ang II + Chl mice (C, D, E) (n = 4–6). *$p < 0.05$ for Ang II vs. CTL, Ang II + Treg cells, and Ang II + Chl. One-way ANOVA followed by Tukey's post hoc test were applied for A–E.

vitro with ER stress (Tudca), autophagy (Chloroquine) and mTOR (Rapamycin) inhibitors. The contractility and relaxation of MRA from control mice were similar before and after incubation with Tudca, chloroquine, and rapamycin (Fig. 2B). However, the incubation of MRA from mice infused with Ang II with Tudca, chloroquine or rapamycin significantly improved endothelial-dependent relaxation (Fig. 2C). We did not observe an effect on the relaxation of MRA isolated from mice infused with Ang II and transferred with Treg cells or treated with chloroquine when incubated in vitro with Tudca, chloroquine, and rapamycin (Fig. 2D, E). Together these data indicate that Treg cells rescued MRA endothelial function through ER stress and autophagy mechanism.

Increased oxidative stress assessed by the enhancement of Nox2, Nox4 expression, and NADPH oxidase activity in mice infused with Ang

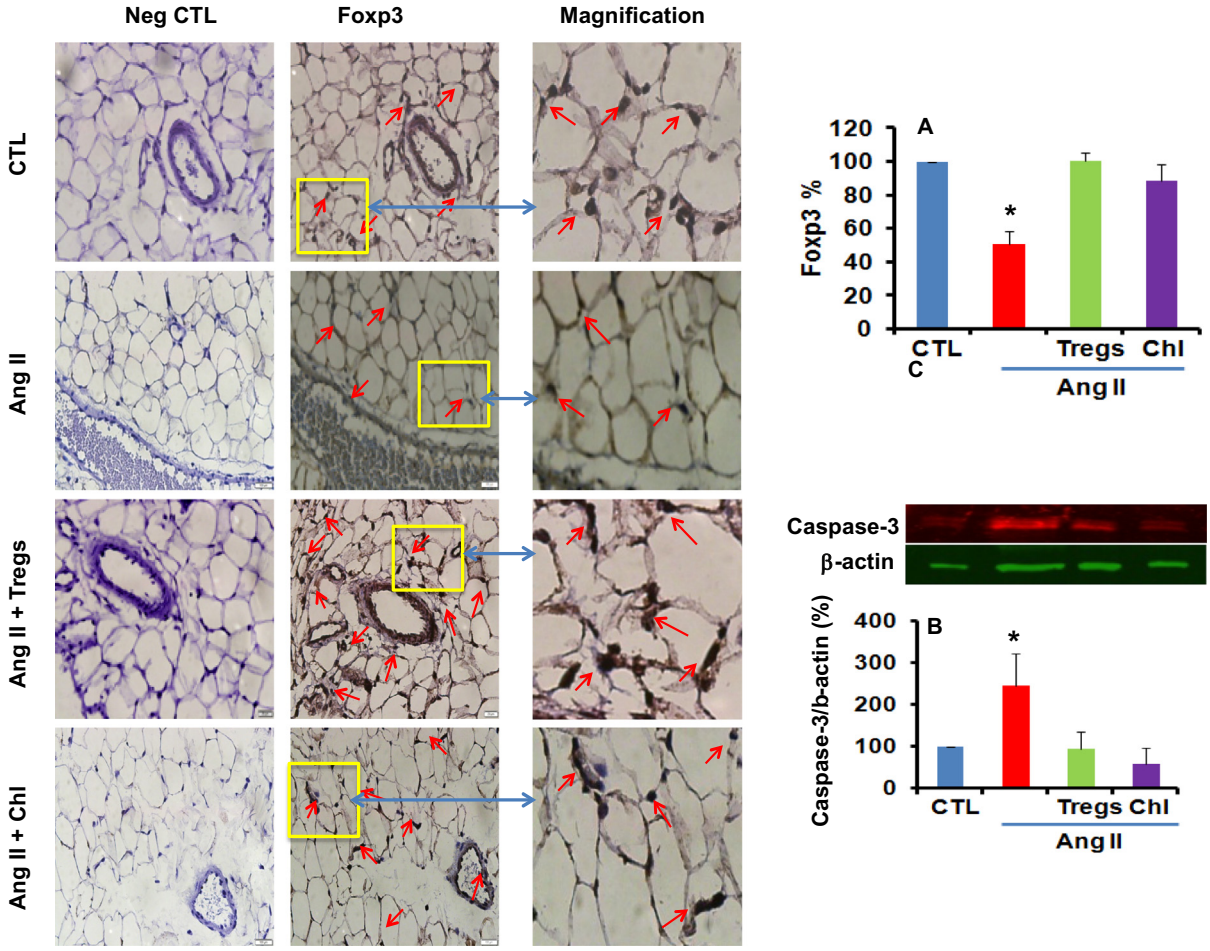

Fig. 6. Treg cells number evaluated by Foxp3 staining in mesenteric resistance arteries (A) of control (CTL), angiotensin II (Ang II), Ang II + Treg cells, and Ang II + Chloroquine (Chl) mice, (n = 3–5), Caspase-3 (B) in Treg cells isolated from spleens of control (CTL), angiotensin II (Ang II), Ang II + Treg cells, and Ang II + Chl, (n = 4), *$p < 0.05$ for Ang II vs. CTL, Ang II + Treg cells, and Ang II + Chl. One-way ANOVA followed by Tukey's post hoc test were applied for A–B.

II was significantly reduced in mice infused with Ang II and transferred with Treg cells or treated with chloroquine (Fig. 3A, B, C). To confirm our data, the NADPH oxidase activity in MRA from mice infused with Ang II was reduced when the samples were incubated with apocynin (Fig. 3C). Vascular oxidative stress induction in mice infused with Ang II was likely through the increase in Nox2 and Nox4 expression, which were reduced with Treg cells transfer and chloroquine treatment These results suggest that Treg cells rescue MRA endothelial function, in mice with established hypertension, through the inhibition of oxidative stress.

Survival and sensor energy pathways were determined in MRA from all groups (Fig. 4A, B, C). The phosphorylated eNOS, Akt, and AMPK levels were reduced in mice infused with Ang II compared to the other groups (Fig. 4A, B, C). We found that Treg cells transfer and autophagy inhibition improved eNOS, Akt and AMPK phosphorylation in MRA from mice with established hypertension (Fig. 4A, B, C).

Autophagy dysfunction plays a pivotal role in the progression of a variety of diseases including cardiovascular complications. Utilizing Western blot analysis, we found that the expression of autophagy markers (Atg5, Beclin1, Atg7) was increased in MRA of mice infused with Ang II, which signifies autophagy dysfunction (Fig. 4D, E, F). These autophagy markers were significantly reduced in mice infused with Ang II and transferred with Treg cells or treated with chloroquine (Fig. 4D, E, F). These results indicate that vascular autophagy dysfunction occurs in established hypertension and is regulated by Treg cells.

ER stress has been documented to be involved in a variety of cardiovascular diseases. Our results revealed that the ER stress induction in the MRA of mice infused with Ang II was blunted with Treg cells transfer and autophagy inhibition (Fig. 4G, H, I). These results suggest that the Treg cells regulate vascular ER stress induction in established hypertension through an autophagy-dependent mechanism. We can suggest that Treg cells transfer improves MRA endothelial function in mice with established hypertension through the inhibition of autophagy and ER stress induction, and the increase in eNOS, Akt, and AMPK activity.

It has been shown that mTOR pathway is activated in hypertension. Here we demonstrated that phosphorylated mTOR levels were elevated in mice infused with Ang II, which were reduced with Treg cells transfer and autophagy inhibition (Fig. 4J). These results suggest that Treg cells and autophagy dictate mTOR activity in hypertension.

Other investigators and we reported that inflammation is a critical factor in vascular dysfunction in hypertension. The results showed an increase in macrophages and leucocytes infiltration in the mesenteric tissue in mice infused with Ang II (Fig. 5A, B), which were reduced with Treg cells transfer and autophagy inhibition (Fig. 5A, B). Our data also showed a decrease in plasma levels of IL-10 and an increase in IL-6 and TNF-α levels in mice infused with Ang II compared to the other groups (Fig. 5C, D, E). The induction of inflammation under established hypertension was associated with a reduction in the number of Treg cell isolated from MRA as shown through the decrease in Foxp3 staining, which was rescued with Treg cells transfer and autophagy inhibition (Fig. 6A).

Western blot analysis demonstrated that established hypertension

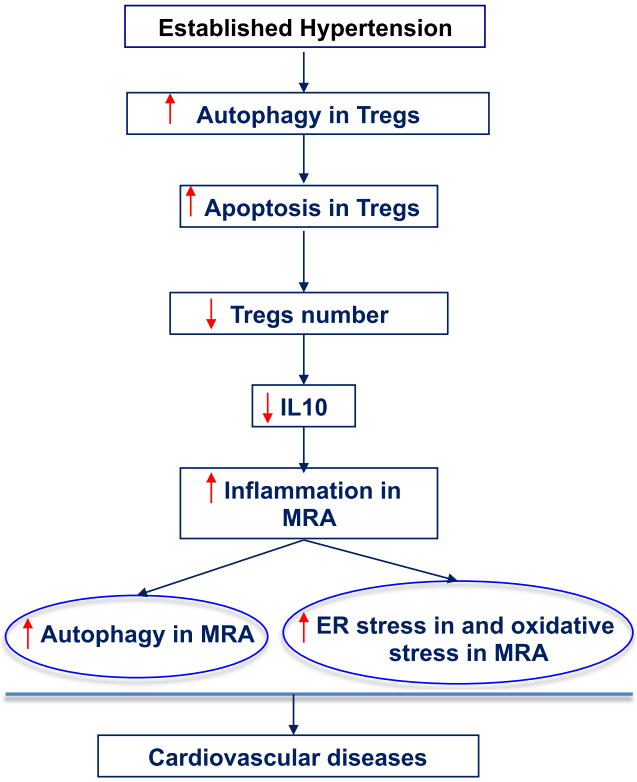

Fig. 7. Summary showing that established hypertension: 1) increases autophagy in Treg cells leading to their enhanced apoptotic rate; 2) reduces Treg cells number and IL-10 level; 3) augments autophagy, inflammation, ER stress, and oxidative stress in mesenteric resistance arteries causing cardiovascular complications.

reduced Treg cells number through an increase in apoptosis mechanism in Treg cells indicated by the higher levels of caspase-3 in Treg cells isolated from mice infused with angiotensin II compared to other groups (Fig. 6B).

Fig. 7 illustrates the summary of the present study showing that established hypertension: 1) increases autophagy in Treg cells leading to their enhanced apoptotic rate; 2) reduces Treg cells number and IL-10 level; 3) augments autophagy, inflammation, ER stress, and oxidative stress in MRA causing microvascular dysfunction.

4. Discussion

The main results of this study indicate that: 1) established hypertension reduced Treg cells number through autophagy dysfunction and apoptosis mechanisms. 2) Treg cells transfer, into a mouse model of established hypertension, rescued the microvascular function, independently of arterial blood pressure lowering effects, through a mechanism that involves the inhibition of autophagy, oxidative stress, ER stress, and inflammation. 3) The inhibition of autophagy also improved the the microvascular function independently of arterial blood pressure.

It is well documented that arterial complications are major risk factors responsible for coronary artery diseases, myocardial infarction, and stroke in patients with established hypertension [26–28]. Clinical observations reported that impaired cardiovascular function and structures predict a poor outcome in patients with cardiovascular diseases [4–6]. The risk of people developing, suffering, and dying from arterial complications related to established hypertension will be significantly reduced after improving arterial function, and structure, and lowering arterial blood pressure. Currently, there are many antihypertensive medications available [7–10]; however, arterial complications associated with established hypertension are still dramatically rising and only controlled in 1/3 of hypertensive patients usually with multiple therapies. Therefore, a novel therapeutic strategy that rescues the cardiovascular physiology in patients with established hypertension is critically needed. Understanding the cellular (the immune Treg and vascular endothelial cells) and molecular mechanisms that cause microvascular complications in established hypertension would substantially impact the public health and have an enormous clinical significance.

Other investigators and we previously reported that Treg cells play an essential role to prevent the development of hypertension and vascular dysfunction [13–15,29]. Prevention approach is challenging, especially when dealing with hypertension known as a silent disease. Thus, we decided to determine whether: 1) autophagy mechanism controls Treg cells survival; and 2) Treg cells could rescue the microvascular function in a mouse model of established hypertension that mimics the clinical situation. Mice were then infused with Ang II for four weeks. By the second week, mice became hypertensive, and then at the beginning of the third week, mice were transferred with Treg cells once or treated with an autophagy inhibitor for 2 weeks. We believe that the fate of transferred Treg cells is similar to the endogenous Treg cells. Data revealed that one single transfer of Treg cells and autophagy inhibition did not affect arterial blood pressure. The results are supported by an earlier study showing that one single transfer of Treg cells into mice infused with Ang II before the development of hypertension does not stop the development of hypertension [12]. However previous studies including ours reported that the transfer of Treg cells six times into mice infused with angiotensin II prevented the development of hypertension [12–15]. These data indicate that one single Treg cells transfer improves the cardiovascular system independently of arterial blood pressure while repetitive transfers of Treg cells reduce blood pressure and improve the cardiovascular system. This concept is crucial and could benefit patients with cardiovascular complications dependent or independent of hypertension.

Hypertension is associated with cardiovascular inflammation and microvascular dysfunction [30–32]. Autophagy is an underlying physiological cellular process which has a significant role in cellular homeostasis maintenance, regulation of ER stress, removing damaged organelles and pathogens [20,21]. Recent evidence suggests that autophagy dysfunction plays a pivotal role in the progression of a variety of diseases including cardiovascular complications [33,34]. Here we showed that established hypertension reduces Treg number through autophagy and apoptosis events. The observed autophagy in Treg cells could be the results of hypertension and/or Ang II. The reduction in Treg cells number induced microvascular inflammation and dysfunction associated with impaired survival pathways (eNOS, Akt, and AMPK)-mechanism. Moreover, autophagy was also increased in mesenteric resistance arteries and that could also participate in the dysfunction of the cardiovascular.

The mesenteric resistance arteries inflammation and dysfunction were rescued in mice with established hypertension transferred with Treg cells or treated with autophagy inhibitor independently of arterial blood pressure. This improvement involves the reduction in autophagy, ER stress, oxidative stress, inflammation, and the increase in eNOS and Akt signaling. All these data correlated with the increase in Treg cells number assessed using Foxp3 staining, and the decrease in caspase 3 expression in Treg cells from mice with established hypertension. We then suggest that Treg cells number declined under established hypertension, through autophagy and apoptosis mechanisms. Interestingly, the transfer of Treg cells and the inhibition of the autophagy rescued the Treg cells number and therefore the microvascular function. Studies showed a correlation between inflammatory cytokines and hypertension [17,35,36]. Our previous studies showed a reduction in inflammation and vascular function improvement in mice infused with Ang II and treated with exogenous IL-10 [14]. Another study demonstrated that Ang II stimulates IL-6 and TNF-α production from various cell types [37], which are the cause of structural and functional

alterations of the cardiovascular system [38]. Treg cells transfer and autophagy inhibition increase IL-10 levels and decrease IL-6 and TNF-α levels indicating the role of Treg cells and autophagy in controlling the inflammation.

A previous study reported that Ang II increased phosphorylated mammalian target of rapamycin (mTOR) [39]. Also, it has been indicated that the inhibition of mTOR reduces cardiac hypertrophy and fibrosis under chronic pressure-overload [40]. These data illustrated that mTOR is an important factor involved in cardiovascular diseases. The data correlated with our results as higher levels of phosphorylated mTOR were detected in the mesenteric resistance arteries of mice infused with Ang II. Interestingly, the transfer of Treg cells and the inhibition of autophagy significantly reduced mTOR activity in mesenteric resistance arteries indicating that Treg cells and autophagy dictate the activity of mTOR.

The important implication from these data is that cell therapy using Treg cells transfer and autophagy inhibition rescued the microcirculation from inflammation and dysfunction independently of arterial blood pressure in established hypertension. Interestingly, the likely increased autophagy in Treg cells promotes apoptosis leading to the reduction in Treg cells number. Thus, we can suggest that the Treg cells are an essential upstream event that regulates the microcirculation function through autophagy mechanism. Therefore, it would be interesting to use Treg cells as cell therapy to overcome established hypertension-induced microvascular pathology.

4.1. Limitation

The transfer of healthy Treg cells or autophagy inhibition rescued the number of Treg cells in the spleen and mesenteric resistance arteries, and reduced autophagy in mesenteric resistance arteries. We think that Treg cells isolated from spleen and mesenteric resistance arteries represent Treg cells in the circulation and tissues. Moreover, it is likely that the rescue of the microvascular functions by chloroquine results from the autophagy inhibition indirectly through protection of Treg cells or directly in mesenteric resistance arteries cells. In vivo experiments are very complex and we believe, as presented in Fig. 7, that the decrease in IL-10 and perhaps the increase in IL-6 and TNFα, both cause inflammation and oxidative stress in mesenteric resistance arteries. Moreover, we recognize the multiplicity of actions of chloroquine and specific knockout of autophagy markers in Treg and vascular endothelial cells will substantially advance the field. A drastic inhibition of autophagy could lead to misfolded protein. In our study we found an increase in autophagy, which was inhibited using chloroquine. We believe that the dose used of chloroquine did not completely stop autophagy.

Transparency document

The Transparency document associated this article can be found, in online version.

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Disclosures

None.

Novelty and significance

What is known?

The immune Treg cells play a significant role in host defense and immune diseases. Other investigators and we reported that Treg cells play an important preventive role in an experimental model of hypertension-induced cardiovascular dysfunction. We also showed that Treg cells regulate oxidative stress in mesenteric resistance arteries. However, whether Treg cells could rescue the mesenteric resistance arteries in a mouse model of established hypertension that mimics the clinical situation is still unknown. Also, the role of autophagy dysfunction in the Treg cells survival and microvascular pathology in established hypertension is not yet understood.

What does new information this article contribute?

Utilizing a mouse model of established hypertension, we determined that the decline in the Treg cells number is through autophagy dysfunction and apoptosis-dependent mechanisms. We delineated that Treg cells transfer into mice with established hypertension rescued the microvascular function through autophagy inhibition independently of arterial blood pressure lowering effect. We also elucidated that the inhibition of autophagy rescued the microvascular function through the decrease in ER stress, oxidative stress, and inflammation, and the increase in survival and energy sensor pathways. Overall, the present study highlights Treg cells as well as the autophagy inhibition as therapeutic strategies to rescue the microcirculation function in established hypertension.

References

[1] M. Olsen, K. Wachtell, C. Meyer, J. Hove, V. Palmieri, H. Dige-Petersen, J. Rokkedal, B. Hesse, H. Ibsen, Association between vascular dysfunction and reduced myocardial flow reserve in patients with hypertension: a LIFE substudy, J. Hum. Hypertens. 18 (2004) 445–452.
[2] A. Coca, F.H. Messerli, A. Benetos, Q. Zhou, A. Champion, R.M. Cooper-DeHoff, C.J. Pepine, Predicting stroke risk in hypertensive patients with coronary artery disease a report from the INVEST, Stroke 39 (2008) 343–348.
[3] M. Bacaner, J. Brietenbucher, J. Labree, Prevention of ventricular fibrillation, acute myocardial infarction (myocardial necrosis), heart failure, and mortality by bretylium: is ischemic heart disease primarily adrenergic cardiovascular disease? Am. J. Ther. 11 (2004) 366–411.
[4] C.A. den Uil, W.K. Lagrand, M. van der Ent, L.S. Jewbali, J.M. Cheng, P.E. Spronk, M.L. Simoons, Impaired microcirculation predicts poor outcome of patients with acute myocardial infarction complicated by cardiogenic shock, Eur. Heart J. 31 (24) (2010) 3032–3039.
[5] J.L. Houghton, E.F. Philbin, D.S. Strogatz, M.T. Torosoff, S.A. Fein, P.A. Kuhner, V.E. Smith, A.A. Carr, The presence of African American race predicts improvement in coronary endothelial function after supplementary L-arginine, J. Am. Coll. Cardiol. 39 (2002) 1314–1322.
[6] S. Tunian Iu, E.E. Mkheian, S.E. Akopov, G.O. Bakunts, A.S. Zograbian, Microcirculatory disorders in patients with cerebrovascular pathology, Zh. Nevropatol. Psikhiatr. Im. S. S. Korsakova 81 (1981) 1316–1320.
[7] W.J. Inder, C. Meyer, P.J. Hunt, Management of hypertension and heart failure in patients with Addison's disease, Clin. Endocrinol. 82 (6) (2015) 789–792.
[8] S. Katayama, Treatment of hypertension in patients associated with type 2 diabetes mellitus or metabolic syndrome, Nihon Rinsho 69 (2011) 2025–2029.
[9] W.S. Aronow, Treatment of systolic and diastolic heart failure in the elderly, J. Am. Med. Dir. Assoc. 7 (2006) 29–36.
[10] G.S. Stergiou, T. Makris, M. Papavasiliou, S. Efstathiou, A. Manolis, Comparison of antihypertensive effects of an angiotensin-converting enzyme inhibitor, a calcium antagonist and a diuretic in patients with hypertension not controlled by angiotensin receptor blocker monotherapy, J. Hypertens. 23 (2005) 883–889.
[11] T.J. Guzik, N.E. Hoch, K.A. Brown, L.A. McCann, A. Rahman, S. Dikalov, J. Goronzy, C. Weyand, D.G. Harrison, Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction, J. Exp. Med. 204 (2007) 2449–2460.
[12] H. Kvakan, M. Kleinewietfeld, F. Qadri, J.K. Park, R. Fischer, I. Schwarz, H.P. Rahn, R. Plehm, M. Wellner, S. Elitok, P. Gratze, R. Dechend, F.C. Luft, D.N. Muller, Regulatory T cells ameliorate angiotensin II-induced cardiac damage, Circulation 119 (2009) 2904–2912.
[13] K. Matrougui, Z. Abd Elmageed, M. Kassan, S. Choi, D. Nair, R.A. Gonzalez-Villalobos, A.A. Chentoufi, P. Kadowitz, S. Belmadani, M. Partyka, Natural regulatory T cells control coronary arteriolar endothelial dysfunction in hypertensive mice, Am. J. Pathol. 178 (2011) 434–441.

[14] M. Kassan, M. Galan, M. Partyka, M. Trebak, K. Matrougui, Interleukin-10 released by CD4(+)CD25(+) natural regulatory T cells improves microvascular endothelial function through inhibition of NADPH oxidase activity in hypertensive mice, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 2534–2542.
[15] A. Leibowitz, A. Rehman, P. Paradis, E.L. Schiffrin, Role of T regulatory lymphocytes in the pathogenesis of high-fructose diet-induced metabolic syndrome, Hypertension 61 (2013) 1316–1321.
[16] D.A. Kasal, T. Barhoumi, M.W. Li, N. Yamamoto, E. Zdanovich, A. Rehman, M.F. Neves, P. Laurant, P. Paradis, E.L. Schiffrin, T regulatory lymphocytes prevent aldosterone-induced vascular injury, Hypertension 59 (2012) 324–330.
[17] E.L. Schiffrin, Immune modulation of resistance artery remodelling, Basic Clin. Pharmacol. Toxicol. 110 (2012) 70–72.
[18] T. Barhoumi, D.A. Kasal, M.W. Li, L. Shbat, P. Laurant, M.F. Neves, P. Paradis, E.L. Schiffrin, T regulatory lymphocytes prevent angiotensin II-induced hypertension and vascular injury, Hypertension 57 (2011) 469–476.
[19] E.L. Schiffrin, T lymphocytes: a role in hypertension? Curr. Opin. Nephrol. Hypertens. 19 (2010) 181–186.
[20] I. Lekli, D.D. Haines, G. Balla, A. Tosaki, Autophagy: an adaptive physiological countermeasure to cellular senescence and ischaemia/reperfusion-associated cardiac arrhythmias, J. Cell. Mol. Med. 21 (6) (2017) 1058–1072.
[21] T. Kanki, K. Furukawa, S. Yamashita, Mitophagy in yeast: molecular mechanisms and physiological role, Biochim. Biophys. Acta 1853 (2015) 2756–2765.
[22] M. Koritzinsky, F. Levitin, T. van den Beucken, R.A. Rumantir, N.J. Harding, K.C. Chu, P.C. Boutros, I. Braakman, B.G. Wouters, Two phases of disulfide bond formation have differing requirements for oxygen, J. Cell Biol. 203 (2013) 615–627.
[23] B.P. Tu, J.S. Weissman, Oxidative protein folding in eukaryotes mechanisms and consequences, J. Cell Biol. 164 (2004) 341–346.
[24] Y. Mei, M.D. Thompson, R.A. Cohen, X. Tong, Autophagy and oxidative stress in cardiovascular diseases, Biochim. Biophys. Acta 1852 (2015) 243–251.
[25] M. Kassan, M. Galan, M. Partyka, Z. Saifudeen, D. Henrion, M. Trebak, K. Matrougui, Endoplasmic reticulum stress is involved in cardiac damage and vascular endothelial dysfunction in hypertensive mice, Arterioscler. Thromb. Vasc. Biol. 32 (2012) 1652–1661.
[26] M.H. Olsen, K. Wachtell, C. Meyer, J.D. Hove, V. Palmieri, H. Dige-Petersen, J. Rokkedal, B. Hesse, H. Ibsen, Association between vascular dysfunction and reduced myocardial flow reserve in patients with hypertension: a LIFE substudy, J. Hum. Hypertens. 18 (2004) 445–452.
[27] A. Coca, F.H. Messerli, A. Benetos, Q. Zhou, A. Champion, R.M. Cooper-DeHoff, C.J. Pepine, Predicting stroke risk in hypertensive patients with coronary artery disease: a report from the INVEST, Stroke 39 (2008) 343–348.
[28] M. Bacaner, J. Brietenbucher, J. Labree, Prevention of ventricular fibrillation, acute myocardial infarction (myocardial necrosis), heart failure, and mortality by bretylium: is ischemic heart disease primarily adrenergic cardiovascular disease? Am. J. Ther. 11 (2004) 366–411.
[29] M.O. Mian, T. Barhoumi, M. Briet, P. Paradis, E.L. Schiffrin, Deficiency of T-regulatory cells exaggerates angiotensin II-induced microvascular injury by enhancing immune responses, J. Hypertens. 34 (2016) 97–108.
[30] P.K. Mehta, K.K. Griendling, Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system, Am. J. Phys. Cell Phys. 292 (2007) C82–C97.
[31] Z.J. Cheng, H. Vapaatalo, E. Mervaala, Angiotensin II and vascular inflammation, Med. Sci. Monit. 11 (2005) RA194–205.
[32] A. Nguyen Dinh, R.M. Touyz Cat, Cell signaling of angiotensin II on vascular tone: novel mechanisms, Curr. Hypertens. Rep. 13 (2011) 122–128.
[33] S. Ghavami, S. Gupta, E. Ambrose, M. Hnatowich, D.H. Freed, I.M. Dixon, Autophagy and heart disease: implications for cardiac ischemia-reperfusion damage, Curr. Mol. Med. 14 (2014) 616–629.
[34] D. Glick, S. Barth, K.F. Macleod, Autophagy: cellular and molecular mechanisms, J. Pathol. 221 (2010) 3–12.
[35] B. Rodriguez-Iturbe, R.J. Johnson, The role of renal microvascular disease and interstitial inflammation in salt-sensitive hypertension, Hypertens. Res. 33 (2010) 975–980.
[36] S.H. Ko, W. Cao, Z. Liu, Hypertension management and microvascular insulin resistance in diabetes, Curr. Hypertens. Rep. 12 (2010) 243–251.
[37] M. Ruiz-Ortega, M. Ruperez, O. Lorenzo, V. Esteban, J. Blanco, S. Mezzano, J. Egido, Angiotensin II regulates the synthesis of proinflammatory cytokines and chemokines in the kidney, Kidney Int. Suppl. (2002) S12–S22.
[38] T. Jayaraman, A. Paget, Y.S. Shin, X. Li, J. Mayer, H. Chaudhry, Y. Niimi, M. Silane, A. Berenstein, TNF-alpha-mediated inflammation in cerebral aneurysms: a potential link to growth and rupture, Vasc. Health Risk Manag. 4 (2008) 805–817.
[39] J.A. Kim, H.J. Jang, L.A. Martinez-Lemus, J.R. Sowers, Activation of mTOR/p70S6 kinase by ANG II inhibits insulin-stimulated endothelial nitric oxide synthase and vasodilation, Am. J. Physiol. Endocrinol. Metab. 302 (2012) E201–E208.
[40] X.M. Gao, G. Wong, B. Wang, H. Kiriazis, X.L. Moore, Y.D. Su, A. Dart, X.J. Du, Inhibition of mTOR reduces chronic pressure-overload cardiac hypertrophy and fibrosis, J. Hypertens. 24 (2006) 1663–1670.