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Research Article

L-arginase induces vascular dysfunction in old spontaneously hypertensive rats

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Keywords:
Hypertension, Arginase, aging, vascular dysfunction, endothelium, Nitric oxide

ABSTRACT

Background: Aging is a major non-modifiable risk factor for hypertension. Changes in aging are similar to those seen in hypertension in the vasculature. Also, aging increases the vascular dysfunction that occurs in hypertension. L-arginase action reduces substrate (L-arginine) availability for the formation of nitric oxide (NO). This reduces the level of NO and leads to reduced vasodilation and ultimately, vascular dysfunction. This study examines the hypothesis that age-dependent vascular dysfunction in SHRs is mediated by arginase. **Methods:** Young (12-14 weeks) and old (11-12 months) male Wistar and spontaneously hypertensive rats (SHR) were used. Mean arterial pressure (MAP) was measured in the rats. They were then euthanized and mesenteric resistance arteries (MRAs) and thoracic aortae were excised and placed in ice-cold physiological salt solution (PSS). Arterial segments were either snap-frozen in liquid nitrogen and stored for immunoblotting studies or cut into 2mm rings for reactivity studies. Cumulative concentration-response curves to acetylcholine (Ach; 10^{-9} – $3x10^{-5}$M) and sodium nitroprusside (SNP; 10^{-12} – $3x10^{-5}$ M) were performed in the absence or presence (30-minute exposure) of L-arginase, 0.05U/ML (MRA) or 0.5U/ML (aorta). Vessels were pre-contracted with phenylephrine (PE; $3x10^{-6}$M) **Results:** MAP increased during aging in the SHRs $p<0.05$ but not in the Wistar rats. Arginase impaired the endothelium-dependent relaxation responses of thoracic aortic and MRA arterial rings to Ach in the old Wistars and SHRs (Emax aorta: 29.42±2.19% vs 7.94±1.86%). Arginase also impaired endothelium-independent relaxation response to SNP in the old SHRs only (Emax aorta: 88.62±4.10% vs 31.45±10.61%). We also observed no differences in the serum arginase activity in the four groups of rats. On the contrary, arginase activity in the aortae of young Wistar rats was reduced compared to other groups. **Conclusions:** Arginase impairs both endothelium-dependent and –independent vasorelaxation responses, through the NO signaling pathway.

INTRODUCTION

Hypertension is a major risk factor for cardiovascular disease frailty. Hypertension is associated with physiological and biochemical changes in the vessel wall, characterized by turbulent blood flow, f luid shear stress, vascular remodeling, and endothelial dysfunction (Mayet and Hughes, 2003).

Elevated arterial blood pressure in most types of hypertension is attributable to increased total peripheral resistance, which results, at least in part, from alterations in humoral and neurogenic components and in vascular endothelial and smooth muscle functions (Schiffrin *et al.,* 2000). Indeed, altered vascular tone, which is a characteristic feature of human and various experimental models of hypertension, has been associated not only with impaired endothelium-dependent vasodilatation and reduced endothelium-derived relaxing factors, including NO, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) signaling, but also with augmented vasoconstrictor signaling (Tang and Vanhoutte, 2010).

Numerous studies have demonstrated the link between the aging process and cardiovascular dysfunction. Aging is a major non-modifiable risk factor for hypertension. The prevalence of hypertension is more than doubled in the elderly than in the young population (Ong *et al.*, 2007). Structural, functional and mechanical changes occur with aging. These changes are similar to those seen in the vasculature in hypertension. Also, aging increases the vascular dysfunction that occurs in hypertension. The characteristic features of vascular dysfunction in hypertension are also present in aging and these include:

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Inflammation, turbulent blood flow, oxidative stress, fluid shear stress, endothelial dysfunction and vascular remodeling (Goeres *et al.*, 2014). Despite the fact that aging is a major risk factor for hypertension, there are relatively less number of research, clinical trials on the treatment of hypertension in older adults; this could be as a result of:

Drug metabolism in the older adults (Sera and McPherson, 2012), Medications and co-morbidities (Benetos *et al.*, 2015) and orthostatic hypotension (Belmin *et al.*, 2000).

Dysfunctional vascular endothelium has been reported to be associated with various forms of human and experimental hypertension (Luscher *et al*, 1987).

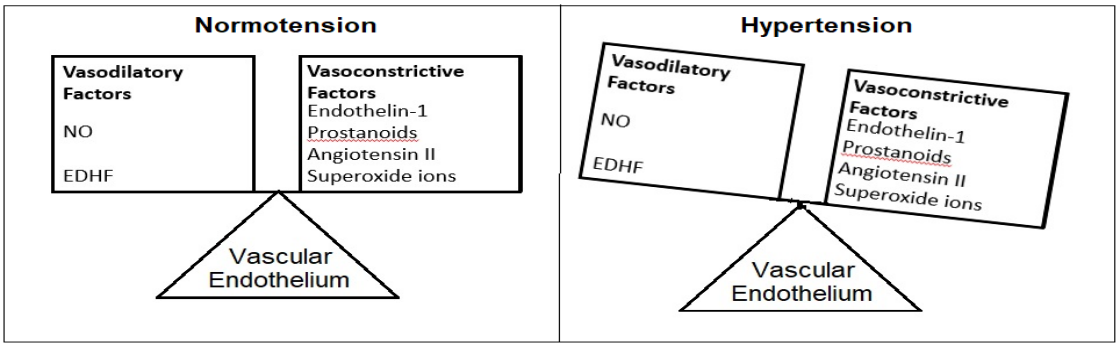

Fig. 1. The role of the endothelium-derived vasoactive substances in the development of hypertension. NO: nitric oxide; EDHF: endothelium derived hyperpolarizing factor.

The endothelial cells release vasoactive factors that modulate vascular tone. NO and endothelium derived hyperpolarizing factors are vasorelaxants released by the endothelium. The endothelium also secretes vaso-constrictive factors including: endothelin1, angiotensin II, and superoxide ions. Thromboxane (Vanhoutte 1989; Sandoo *et al.*, 2010). Under physiological conditions, there is a balance between vasoconstrictive and vasorelaxants factors released by the endothelial cells. This balance is altered in hypertension and this leads to endothelial dysfunction, decrease in NO production and vasodilation (Versari *et al.*, 2009). Different mechanisms have been proposed for the decreased NO seen in hypertension. As shown in figure 1, in normal physiological conditions, the endothelium secretes vasoconstrictive and vasodilatory substances, but during hypertension, this balance is tilted therefore, the endothelium secretes more of vasoconstrictive substances, which leads to increased vasoconstriction and reduced vasorelaxation.

Arginase is a ureohydrolase enzyme that converts L-arginine into L-ornithine and urea. Two isoforms of arginase have been cloned (Haraguchi et al., 1987). Its presence in the liver was first described by Krebs and Henseleit in 1929 (Jenkinson et al., 1976) and crude preparation were reported as far back as 1931 (Salaskin and Solowjew 1931, and Waldschmidt-Leitz et al., 1931). In 1956, the "partial" purification was further improved by Robbins and Shields. (Robbins and Shields 1956). A study by Buga et al., (1996) reveals for the first time that substantial arginase activity is present constitutively in rat aortic endothelial cells. The presence of arginase I and II as well as their activity and expression in cultured vascular smooth muscle and endothelial cells have been reported (Zhang et al., 2001; Johnson et al., 2005). Upregulation of arginase has been reported to be associated with aging and cardiovascular diseases (Toque et al., 2013). Studies by Johnson et al, 2005, showed that enhanced vascular arginase activity contributes to endothelial dysfunction in Dahl-S rats with salt-induced hypertension.

The roles of arginases in vascular disease, pulmonary disease, infectious disease, and cancer have been studied (Morris 2002, Santhanam et al. 2008,).

Radiometric assays showed that in pathological conditions, arginase, compared to eNOS, is, at baseline, the major metabolic pathway for l-arginine utilization in cell extracts (Bachetti et al., 2004).

Arginase inhibitors have been used to probe the role of arginase in the regulation of NO-mediated smooth muscle relaxation in the gastrointestinal tract as well as in penile and clitoral corpus cavernosum tissues (Kim et al., 2001). Furthermore, the endothelial dysfunction observed with various forms of hypertension can be reversed by the administration of L-arginine (Chen and Sanders 1991; 1993; Hu and Manning, 1995). Increased arginase expression has been reported in hypertensive disorders and is associated with decreased NO bioavailability and enhanced vascular reactivity (Johnson *et al*, 2005; Demougeot *et al*, 2005).

Arginase action reduces substrate (L-arginine) availability for the formation of nitric oxide (NO). This reduces the level of NO and leads to reduced vasodilation and ultimately, vascular dysfunction. This

study tests the hypothesis that the age dependent vascular dysfunction in spontaneously hypertensive rats (SHRs) is mediated by arginase.

METHODS

Animals:

Young (12-14 weeks) and old (11-12 months) male Wistar and spontaneously hypertensive rats were used in this study. They were purchased from Envigo RMS, Inc. and all rats were maintained in GM500 individually ventilated cages (Animal Care Systems), at 21°C, 50–70% humidity, on a 12-hour light/dark cycle, chow and water were made available *ad libitum*. All animal handling procedures were performed in accordance with the Guide for the Care and use of Laboratory Animals of the National Institutes of Health (NIH) and were reviewed and approved by the Institutional Animal Care and Use Committee of Augusta University.

Blood pressure measurements:

Mean arterial pressure (MAP) was measured in the rats under anaesthesia (isoflurane 1.5%, via inhalation). The animal was carefully dissected to expose the femoral artery; the catheter was inserted into the femoral artery while the other end was connected to the pressure transducer coupled to a Powerlab system (Powerlab 4SP/ML750). They were then euthanized and 5ml of blood was drawn from the heart to obtain serum for the assay of arginase activity.

Vascular function

Mesenteric resistance arteries (MRAs) and thoracic aortae from young and old wistar and Spontaneously hypertensive rats (SHRs) were excised and placed in 4˚C Physiological salt solution (PSS) containing (mmol/L): NaCl (130), $NaHCO_3$ (14.9), KCl (4.7), KH_2PO_4 (1.18), $MgSO_4{\cdot}7H_2O$ (1.18), $CaCl_2{\cdot}2H_2O$ (1.56), EDTA (0.026), and glucose (5.5) (all Sigma-Aldrich). Thoracic aortae and second order MRAs were carefully cleaned of adhering perivascular adipose tissues under light microscopy in ice-cold PSS and cut into 2mm segments, divided into two groups; one group of arterial segments was denuded of their endothelium. The MRA endothelium was denuded by rubbing the lumen with a hair shaft (McCarthy *et al.*, 2018) while the aorta was denuded by gently rubbing the lumen with tweezers. The MRA rings were mounted on DMT wire myographs (Danish Myo Tech, Aarhus, Denmark) and were normalized to their optimal lumen diameter for active tension development which was determined based on the internal circumference (L_0) t0 90% of what the vessels would have if they were exposed to a passive tension equivalent of 100mmHg (L_{100}) transmural pressure (Mulvany and Halpern, 1976). The diameter (I_1) was then determined according to the equation $I_1 = L_1/\pi$, using the software specific for normalization of resistance arteries (DMT Normalization Module; LabChart v.5.5.6, AD Instruments). The aortic rings were mounted on DMT pin myograph (Danish Myo Tech, Aarhus, Denmark) and tension was set to a basal force of 30mN. Arteries were then bathed in PSS maintained at 37˚C, bubbled continuously with 5% $CO_2$and 95% $O_2$for 30 minutes and rinsed three times at 10-minute intervals. Thereafter, the vessels were initially contracted with 120mM KCL PSS (the vessels were considered viable only if they contracted to a force greater than 10mN in response to 120mM KCL). Endothelium integrity was then tested by contracting the MRA rings with $3x10^{-6}$M Phenylephrine (PE) followed by relaxation with $3x10^{-6}$ M Acetylcholine (ACH) and the aortic ring endothelium denudation was considered successful if arterial rings relaxed less than 25% to Ach (Ebeigbe and Aloamaka, 1985). Arterial rings were washed with fresh PSS three times and rested for 10 minutes after which they were subjected to one of the following protocols:

Experimental Protocols:

Evaluation of endothelium-dependent relaxation responses

MRAs and aortae with confirmed intact endothelium were initially contracted with PE ($3x10^{-6}$M); when the contraction attained a plateau, cumulative concentration-response tests to ACH (10^{-9}-10^{-5}M) were performed. Thereafter, the rings were washed thrice with fresh PSS and allowed to rest for 20 minutes. Subsequently, the above relaxation-response protocol was repeated following incubation of the rings for 30 minutes with either 0.05U/ML (MRA) or 0.5U/ML (aorta) L-arginase.

Evaluation of endothelium-independent relaxation responses

MRAs and aortae with confirmed denuded endothelium were initially contracted with PE (10^{-6}M); when the contraction attained a plateau, cumulative concentration-response tests to SNP (10^{-12}-10^{-5}M) were performed. Thereafter, the rings were washed thrice with fresh PSS and allowed to rest for 20 minutes. This was followed by a repeat of the above relaxation-response tests in rings incubated for 30 minutes, with either 0.05U/ML (MRA) or 0.5U/ML (aorta) L-arginase.

Arginase activity Assay

Arginase activity was measured using the arginase activity assay kit (catalog number: MAK112) purchased from Sigma-Aldrich (St. Louis, MO, USA). It provided a simple and direct protocol for measuring arginase activity in the serum and tissues.

Immunoblotting

Thoracic aortae and MRAs were cleaned of perivascular adipose tissue and═snap-frozen in liquid Nitrogen. Expression of arginase, and eNOS (endothelial nitric oxide synthase), were measured. Arterial segments were homogenized in a cold protein extraction buffer. Equal amounts of protein were separated using 6, 10 or 15% SDS-PAGE. Gels were transferred to nitrocellulose membranes and standard immunoblotting procedures were done using primary antibodies indicated above. Immunoreactive bands were visualized with the enhanced chemiluminescence detection system and quantified using Alpha Imager software. β-actin was used to normalize expression.

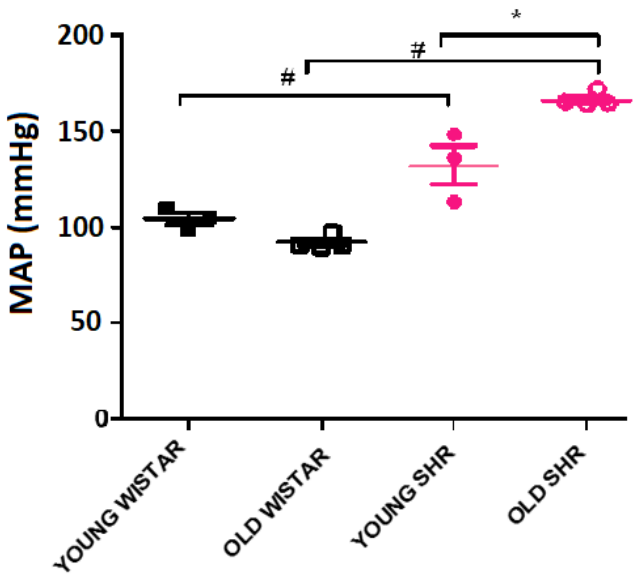

Fig. 2: Mean arterial blood pressure (mmHg) of young and old SHR and Wistar rats. Values presented as mean ±SEM of 6 rats in each group.***** p<0.05 compared to young SHR; # p<0.05 compared to age-matched Wistars

Statistical Analysis

Data are expressed as means ± S.E.M. of 6 rats per group; statistical differences were calculated using Student's t-test, one-way and two-way ANOVA with repeated measures followed by Bonferroni post hoc test. Significance was set at p<0.05. All statistical tests were performed using Graphpad Prism (v. 6.0 Graphpad software).

RESULTS

Blood Pressure of Rats

We observed a significant difference in the MAP (mmHg) of the young SHRs compared to the young wistar rats (132 ± 10.25 vs 104.4 ± 3.012 p<0.05); the old SHRs compared to the old wistar rats (166.2 ± 1.9 vs 92.08 ± 1.545 p<0.05) and between the old and young SHRs (166.2 ± 1.9 vs 132.3 ± 10.25 p<0.05), but no difference was observed between the young and old wistar rats (104.4 ± 3.012 vs 92.08 ± 1.545).

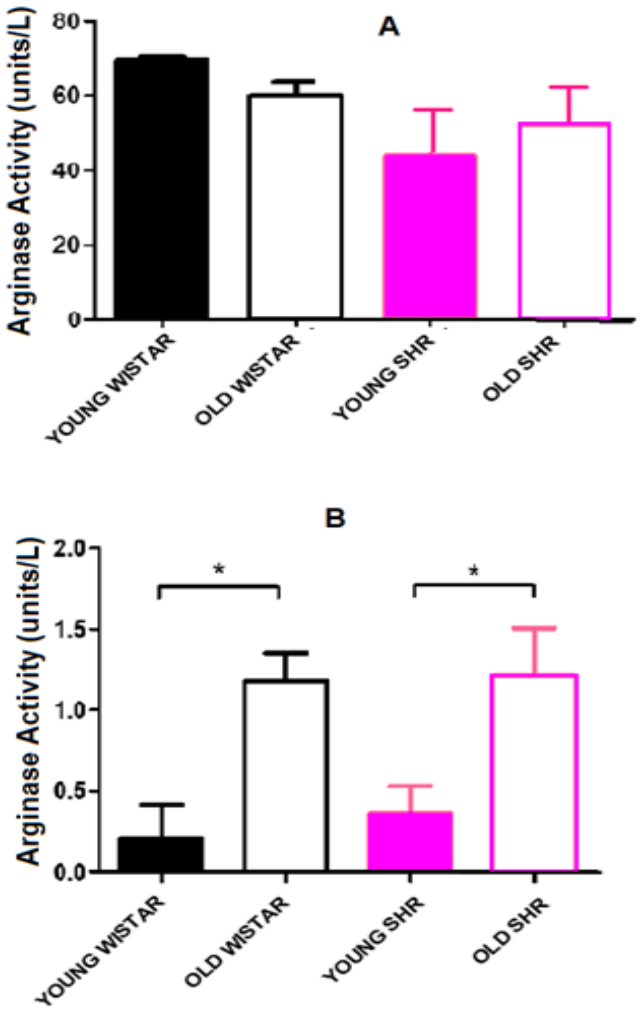

Fig. 3: Evaluation of arginase activity in serum (A) and thoracic aortae (B) of young and old wistar rats and SHR. Mean ± S.E.M. of 6 rats. * p<0.05 compared to respective controls using one-way ANOVA with Bonferroni post hoc test.

Serum Arginase Activity

Serum arginase activity was not significantly different in the four groups of rats; however, aortic arginase activity was increased in the old Wistars and SHRs.

Western Blot Studies

The expression of arginase 1 and eNOS was examined in the thoracic aortae of the groups of rats, to determine if it there will be differences between the groups of the rats. Arginase expression was lower in the young wistar aortae compared to other groups. There were no differences in the eNOS expression in these groups of rats.

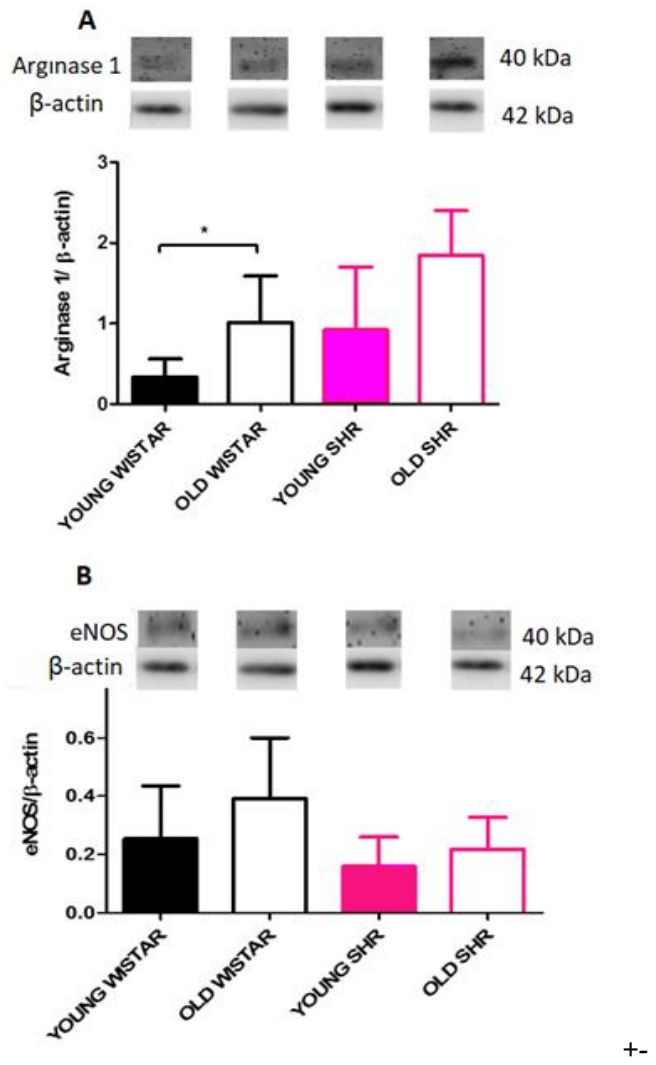

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Fig. 4: Assessment of arginase 1(A) and eNOS (B) expression in the thoracic aortae of the groups of rats. Arginase expression was lower in the young wistar aortae compared to other groups. There were no significant differences in the eNOS expression in these groups of rats. Mean ± S.E.M. of 4 rats. * p<0.05 compared to control using one-way ANOVA with Bonferroni post hoc test.

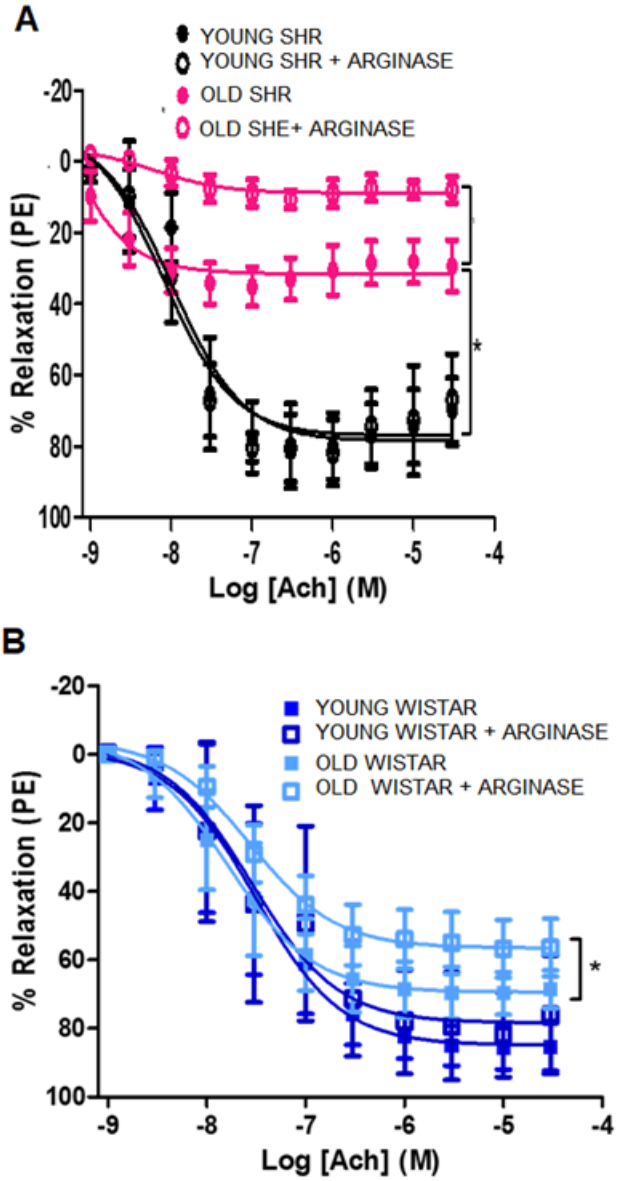

Fig. 5: Evaluation of concentration dependent relaxation responses of thoracic aortic ring segments from young and old rats to ACH [10^{-9} – $3x10^{-5}$ M] before and after incubation with L-Arginase [0.5U/ML] for 30 minutes. A: SHR, B: Wistar rats. Vessels were pre-contracted with 3µM phenylephrine. Mean ± S.E.M. of 6 rats ***** p<0.05 compared to controls.

Concentration Dependent Responses to Acetylcholine in Arterial Ring Segments of Young and old SHR and Wistar Rats

To test our hypothesis that age dependent vascular endothelial dysfunction is mediated by arginase, concentration dependent relaxation responses of young and old SHR and wistar thoracic aortic rings and MRA segments to ACH [10^{-9} – $3x10^{-5}$ M] before and after incubation with L-Arginase [0.5U/ML] for 30 minutes were evaluated. We observed a significant decrease in concentration dependent relaxation response to ACH [10-9 – 3x10-5 M] in the old SHR rings incubated with L-arginase compared with the rings without incubation (p<0.05); but no significant difference was observed in the young.

Concentration Dependent Responses to Sodium Nitroprusside in Arterial Ring Segments of young and old SHR and Wistar Rats

In order to test if the vascular dysfunction mediated by Arginase is only in the endothelium, the endothelium was denuded and the concentration dependent relaxation responses of thoracic aortic and mesenteric resistance arterial ring segments to SNP [$3x10^{-12}$ – $3x10^{-5}$ M] before and after incubation with L-Arginase [0.5U/ML] for 30 minutes were evaluated. Vessels were pre-constricted with 3µM phenylephrine. Mean ± S.E.M. of 6 rats ***** p<0.05 compared to old SHR. We observed a

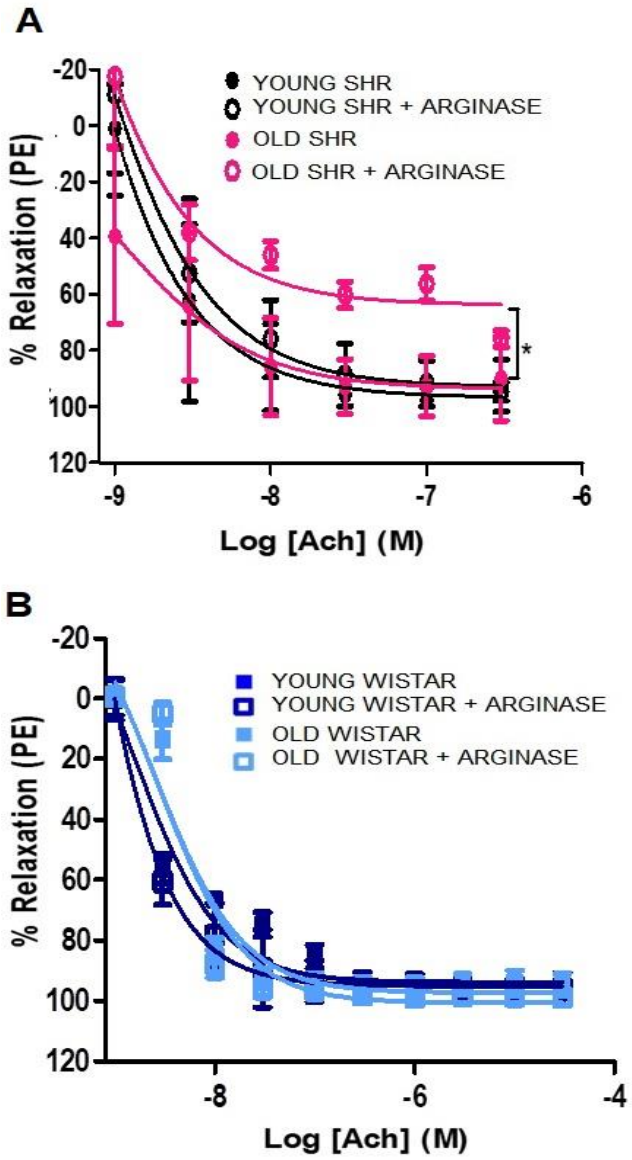

Fig 6: Evaluation of concentration dependent relaxation responses of mesenteric resistance arterial ring segments from young and old rats to ACH [10^{-9} – $3x10^{-5}$ M] before and after incubation with L-Arginase [0.5U/ML] for 30 minutes. A: SHR, B: Wistar rats. Vessels were pre-contracted with 3µM phenylephrine. Mean ± S.E.M. of 6 rats ***** $p<0.05$ compared to controls.

significant decrease in concentration dependent relaxation response to SNP [$3x10^{-12}$ – $3x10^{-5}$ M] in the old SHR rings incubated with L-arginase compared with the rings without incubation ($p<0.05$). No difference was observed in all other groups.

DISCUSSION

Studies have reported that the serum level of arginase is increased in some disease conditions and this could be a diagnostic tool in the prognosis of such diseases (Pernow and Jung, 2013). Studies, using animal models of hypertension have reported increases in arginase activity (Gudi *et al.*, 1996; Rodriguez *et al.*, 2000; Zhang *et al.*, 2004; Johnson *et al.*, 2005; Demougeot *et al.*, 2007;) in the arteries of these animals.

There were no significant differences in the serum arginase activity in the four groups of rats, but arginase activity in the thoracic aorta of young wistar was reduced compared to the other groups.

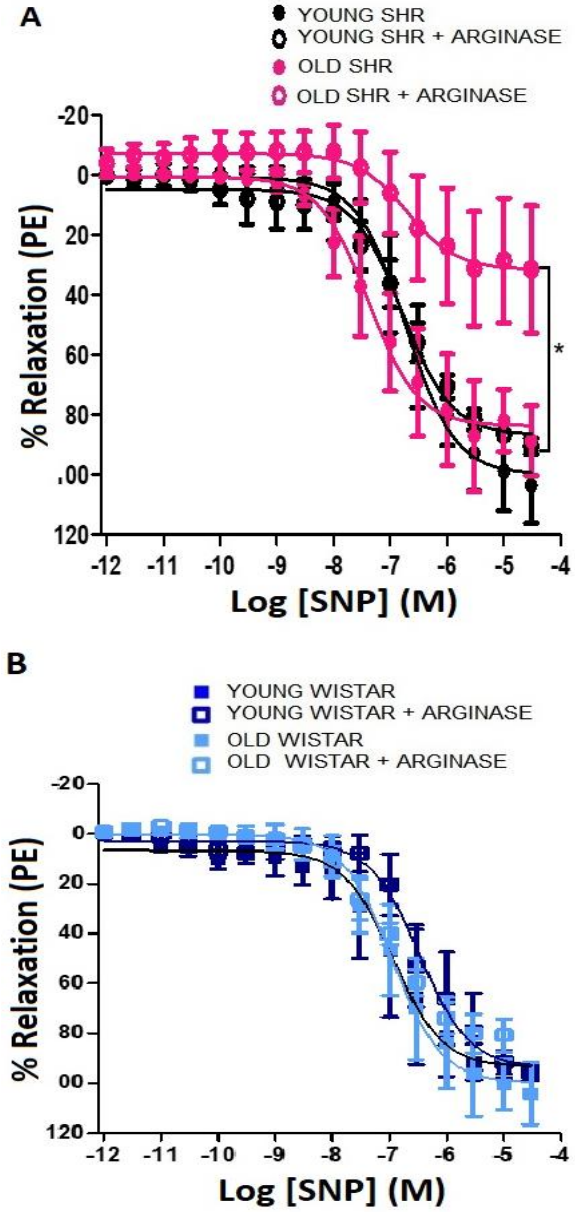

Fig. 7: Evaluation of concentration dependent relaxation responses of thoracic aortic arterial ring segments from young and old rats to SNP [$3x10^{-12}$ – $3x10^{-5}$ M] before and after incubation with L-Arginase [0.5U/ML] for 30 minutes. A: SHR, B: Wistar rats. Vessels were pre-contracted with 3µM phenylephrine. Mean ± S.E.M. of 6 rats. *$p< 0.05$ compared to the other groups.

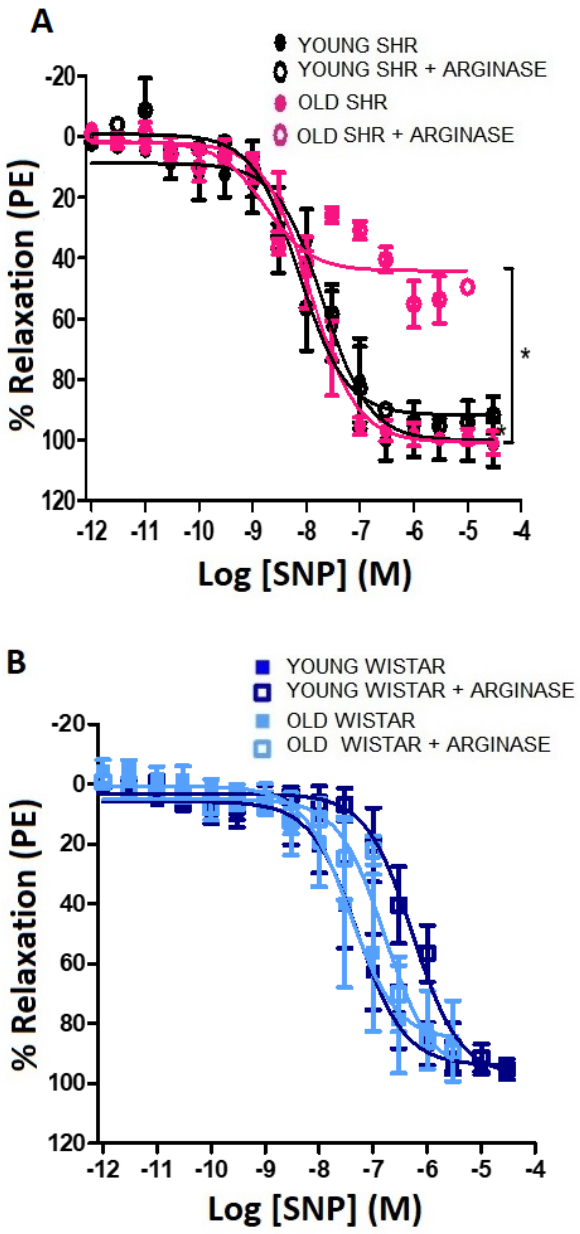

Fig. 8: Evaluation of concentration dependent relaxation responses of mesenteric resistance arterial ring segments from young and old rats to SNP [$3x10^{-12}$ – $3x10^{-5}$ M] before and after incubation with L-Arginase [0.5U/ML] for 30 minutes. A: SHR, B: Wistar rats. Vessels were pre-contracted with 3µM phenylephrine. Mean ± S.E.M. of 6 rats. *p< 0.05 compared to the other groups.

There were no significant differences in the serum arginase activity in the four groups of rats, but arginase activity in the thoracic aorta of young wistar was reduced compared to the other groups. Increased arginase activity has been reported in arteries of animal models of hypertension (Rodriguez *et al.*, 2000; Johnson *et al.*, 2005; Demougeot *et al.*, 2007; Bagnost *et al.*, 2009). Our results showed that arginase expression was less in the young wistar compared to other groups. This is consistent with literature that arginase is expressed more in aging and hypertension.

ACh acts on the muscarinic receptors of the endothelial cells and causes a release of nitric oxide that results in relaxation of the vascular smooth muscles. This relaxation of arterial smooth muscle by ACh is dependent on the presence of the endothelium (Furchgott and Zawadzki, 1980). They observed that removal of the endothelium by rubbing the internal surface of the blood vessels led to impaired Ach induced relaxation.(Furchgott and Zawadzki, 1980).

We examined the effect of arginase on the Ach induced endothelium dependent relaxation in young and old normotensive and hypertensive rats pre-contracted with 3µM phenylephrine. Arginase competes with eNOS for L-arginine, the substrate for nitric oxide production and reduces the availability of nitric oxide, thereby reducing the relaxation of the vascular smooth muscle (Durante *et al.*, 2007). This attenuation in vascular relaxation to Ach was observed in the thoracic aortic and mesenteric resistance arterial ring segments of old wistar and spontaneously hypertensive rats. This indicates that arginase inhibited the production of NO by eNOS in the endothelium of the old rats by using up all the L-arginine. Arginase is broken down to release urea and L-ornithine (Ash, 2004). Urea is readily excreted by the kidney, but L-ornithine is broken down by Ornithine decarboxylase to polyamines and Ornithine amino transferase to L-proline. Polyamines have been shown to impair endothelium dependent vasoconstriction (Tabor and Tabor, 1984). On the other hand, arginase had no effect on the endothelium dependent relaxation responses to ach in the thoracic aortic and mesenteric resistance arterial ring segments of young wistar and spontaneously hypertensive rats. This may be as a result of the abundance of L-arginine in the endothelium of the arterial rings of these young animals, so even in the presence of arginase, there was enough substrate for eNOS to convert to NO.

To test if the vascular dysfunction mediated by Arginase is endothelium-dependent, we denuded the endothelium and evaluated the concentration dependent relaxation responses of thoracic aortic arterial ring segments to sodium nitroprusside (SNP). SNP releases NO in the circulation by binding to oxyhaemoglobin to release NO. It therefore supplies NO for the relaxation of the vascular smooth muscle (Friederich *et al.*, 1995). The NO supplied by SNP diffuses into the vascular smooth muscle cells to cause vasorelaxation; therefore, the relaxation responses to SNP are not endothelium dependent. We observed that arginase attenuated vascular relaxation to SNP in the thoracic aortic and mesenteric resistance arterial ring segments of old spontaneously hypertensive rats. On the other hand,

arginase had no effect on the endothelium independent relaxation responses to SNP in the thoracic aortic and mesenteric resistance arterial ring segments of young and old Wistar and young spontaneously hypertensive rats. These observations suggest that arginase in addition to inducing endothelial dysfunction also induces vascular smooth muscle dysfunction the old hypertensive rats. The impaired relaxation to SNP in the old SHRs is indicative of possible arginase induced impairment of the downstream signaling pathway of NO-mediated relaxation.

CONCLUSION

The results of this study suggest that arginase impairs both endothelium-dependent and –independent vasorelaxation responses, through the NO signaling pathway.

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