Theoretical Medical Sciences Ph.D. Program

AGE-DEPENDENT CHANGES IN ANGIOTENSIN II-INDUCED ARTERIAL VASOCONTRACTION AND VASCULAR AT_1-RECEPTOR EXPRESSION

Ph.D. Thesis

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PÉCS, 2015

1. INTRODUCTION

Angiotensin II (Ang II) by activating angiotensin type 1 receptors (AT_1R) is one of the most potent vasoconstrictors in the regulation of vasomotor tone and thus systemic blood pressure. The topic of this thesis is the examination of age related changes in angiotensin II (Ang II) induced vasomotor activity, AT_1-Receptor (AT_1R and AT_2R) mRNA and AT_1R protein expression. In developing countries the aging and age-dependent physiological and pathological changes are important public health problem in modern society. Previous studies described the link between age-related molecular, cellular (Ungvari, 2011), and functional changes that occur in the cardiovascular system (Ungvari, 2012, Valcarcel-Ares 2012). These are important because the age-associated arterial structural and functional changes are closely linked to an impairment of coronary and cerebromicrovascular blood flow and development of cardiac ischemia and vascular cognitive impairment (Ungvari, 2013). With aging, the altered responsiveness of vessels and expression levels of the AT_1R and AT_2R mRNA level have been observed, but more details about the meaning and potential mechanism of the change of AT_1R and AT_2R expression during aging from young to senescence require further investigations (Wang, 2003) in order to understand the potentials of systemic and local renin–angiotensin system to regulate vasomotor functions. By now it is well established that aging reduces the dilator function of vessels, thereby contributing to the development of vasomotor dysfunction and dysregulation of peripheral vascular resistance (Diz, 2008). Much less is known regarding the age-dependent changes in the contractile functions of vessels, such as Ang II-induced vasomotor responses. This issue is important because Ang II plays an important role in the regulation of tissue blood flow peripheral vascular resistance, and thus blood pressure (Marin 1999). Previous studies suggested that aging decreases the contractile response of isolated rat thoracic aorta to Ang II (Tschudi, 1995). Other studies showed an age-dependent increase in the Ang II-induced contractile responses in isolated coronary arteries of spontaneously hypertensive rats; however, there were no differences observed in normotensive Wistar Kyoto rats from 1- to 18-month-old (Tschudi, 1995). Whereas others found that in mesenteric arteries, the Ang II-induced constrictions diminished with aging from 1- to 8-month-old rats (Konishi 1997). These findings appear to be controversial and could be due to several reasons: among others, many of these results have been obtained in different ages and species of animals and vessel preparations (Konishi 1997), and the experimental protocols were also different (varying in the length–tension curves, incubation time, oxygen concentration of bath chambers and so on). In addition, it is difficult to assess the magnitude of Ang II-induced responses of isolated vessels due to tachyphylaxis, namely the continuous reduction in Ang II-induced contractions after repeated administrations (Bagi, 2008, Leite, 1997). The primary vasomotor actions of Ang II have been attributed to the stimulation of the AT_1R, which exhibit desensitization as shown by the reduced constriction to sequential application of Ang II (Bagi, 2008). Interestingly, AT_1Rs are highly expressed in rat fetal brainstem tissues, which decreases during maturation; however, in whole kidney tissue, AT_1R expressions do not change between newborn

and senescence age (28 months) in Sprague–Dawley rat (Li, 2010). These data show that AT_1R can be differentially expressed under different developmental tissue- and disease-specific conditions (Li, 2010). Although numerous studies have shown that age is a dominant risk factor for cardiovascular diseases in association with the alterations in the vasomotor function of large arteries (Marin, 1999), at present, very little is known how aging modulates the vasomotor response to Ang II and the vascular expression of mRNA level and protein AT_1R and AT_2R-mRNA expression (Marin, 1999). These issues are important however, because Ang II is one of the key multiple role signalling molecules of the renin-angiotensin system responsible for the regulation of cardiovascular system, both in health and diseased conditions (Paravicini, 2008).

2. HYPOTHESIS

In the present study, we hypothesized that aging has a major impact on the mean arterial blood pressure (MABP), on magnitude and characteristic of Ang II-induced contractile responses-curves of isolated arterial vessels, which correlate with the vascular AT_1R-mRNA and AT_1-protein expression, but not with AT_2R-mRNS expression. Also, that the AT_1R are mostly present in the smooth muscle layer of the carotid arteries.

Thus, we characterized the vasomtor responses of isolated rat carotid arteries to repeated administrations of Ang II and vascular AT_1R and AT_2R mRNA and AT_1R protein expressions as a function of age, from newborn to senescence. For this study, we have chosen carotid arteries because experimental and clinical studies documented that carotid arteries mirror the changes occurring in coronary and cerebral vessels (Rothwell, 2001).

3. MATERIALS AND METHODS

3.1. Experimental animals and their housing

Different ages of male Wistar Kyoto rats were used: 8 days, 1, 2, 6, 9, 12, 24, and 30 months (body weights: 14±4 g (n=5), 108±14 g, 202±15 g (n=5), 281±15 g (n=5), 320±10 g (n=5), 292±18 g (n=5), 360±14 g (n=5), and 270±54 g (n=5). Rats were kept individually in plastic cages with approximately 3-5 cm wood shaving bedding. Food pellets were available ad libitum with the exception of fasting periods, when only tap water was provided. The experiments were run according to the general rules set in the Hungarian law on animals and the experimental protocols used were approved by the Ethical Committee of the Pécs University (BA02/2000–8/2008 directive).

3.2. Surgeries

Rats were operated under ketamine/xylazine anesthesia (78 mg/kg Calypsol [Richter] + 13 mg/kg [Eurovet]). The anesthesia was always the same regardless of the type of the surgery. After anesthesia, the common carotid arteries were isolated from the rats, cleaned, and prepared under an Olympus operation microscope and quickly transferred

into an ice/cold (4°C) oxygenated (95% O_2, 5% CO_2) physiological Krebs solution, as described previously (Veresh, 2012). Then, the carotid arteries were dissected into 5-mm rings.

3.3. Measurement of arterial blood pressure

Rats were anesthetized with intraperitoneal ketamine + xylazine anesthesia (78 mg/kg calypsol [Richter] + 13 mg/ kg [Eurovet], respectively) and placed on a heated pad to maintain body temperatures at 37°C. The left carotid artery was cannulated by a polyethylene catheter that was connected to a pressure transducer (Experimetria, Hungary), and the blood pressure was measured and monitored online by a data acquisition computer system (ISOSYS, Experimetria, Budapest, Hungary), and the mean arterial blood pressure was calculated.

3.4. Measurement of isometric force of isolated rat carotid arteries in response to Ang II

Each ring was positioned between two stainless steel wires (diameter 0.04 mm) in a 5-mL organ bath of a wire myograph system (DMT 610M, Danish Myo Technology, Aarhus, Denmark). Isometric tension generated by the vessels was continuously measured, and the software Myodaq 2.01 M610+ was used for data acquisition and display. At the beginning of experiments, the length–tension curve—normalized to 2.0 g (13.34 mN)—was obtained, and the vessels were allowed to -stabilize for 60 minutes before experimental protocols (Allen, 1999). At the beginning and the end of experiments, administration of 60 mM KCl was used to assess the vasomotor capability of carotid arteries (Paravicini, 2008). The bath solution was continuously oxygenated with a gas mixture of 95% O_2 plus 5% CO_2, and kept at 36.8°C (pH 7.4). In the first series of experiments, increasing doses of Ang II (10–9 to 10–5 mol/L) were administered to the vessels bath. Two dose–response curves to Ang II were obtained in a sequential manner (1-administrations and 2-administrations). In preliminary studies, we determined the optimal time delay between the two responses. We have found that 20 minutes was necessary for tachyphylaxis to develop (Bagi, 2008). In a group of experiments, the endothelium was removed by hair (Diz, 2007), and vasomotor responses were obtained in the presence and absence of endothelium. The functional presence or absence of endothelium was tested by the vasomotor responses to acetylcholine (Bagi, 2008). The vasomotor curves were charachterised by time measurment (time from „baseline to peak", time frome „peak to baseline")

3.5. Measurement of mRNA level of vascular AT_1R and AT_2R

To assess the expression of AT_1R and AT_2R in carotid arteries, mRNA extraction and quantitative reverse-transcriptase polymerase chain reaction were used. Total RNA extraction from the right and left carotid arteries of rats (aged from 8 day to 30 months) and reverse transcription of RNA (0.5 μg) were performed as described previously (Zhang, 2008). One microliter of complementary DNA was used in the reactions 45 × 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds preceded by an initial 95°C for 10 minutes. Downloaded from with Maxima SYBR

Green/Fluorescein qPCR Master Mixn(Fermentas, Burlington, Ontario, Canada). Detection was performed with the Chromo 4 System (Bio-Rad, Hercules, CA, USA). The following primers were used:

Receptor / Control	Primer
AT_1	Fwd 5'-GGTTCAAAGCCTGCAAGTGAA-3'
	Rev 5'-GAGTGAGCTGCTTAGCCCAAA-3'
AT_2	Fwd 5'-CAATCTGGCTGTGGCTGACTT-3'
	Rev 5'-TGCACATCACAGGTCCAAAGA-3'
18S rRNS	Fwd 5'-TTAAGTCCCTGCCCTTTGTACAC-3'
	Rev 5'-GATCCGAGGGCCTCACTAAAC-3'

Statistical analysis of relative expression of the target gene was based on the ΔΔCt-method with efficiency correction made with the program Opticon Monitor Version 3.1 (Bio- Rad, Hercules, CA, USA) normalized for the housekeeping gene 18S rRNA as previously.

3.6. Measurement of protein level of vascular AT_1R

According to previous studies (Bartha, 2009), segments of carotid arteries were homogenized in ice-cold 50 mM Tris-buffer, pH 8.0 containing protease inhibitor cocktail 1:1000 and 50 mM sodium vanadate (Sigma–Aldrich Co., Budapest) and harvested in 2 x concentrated sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer. Proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide electrophoretic gels. Proteins were transferred to Protran nitrocellulose membranes. After blocking (2 hours with 3% nonfat milk in Tris-buffered saline), membranes were probed overnight at 40°C with antibodies recognizing AT_1R (1:500; Santa Cruz Biotechnology Inc.). Membranes were washed six times for 5 minutes in Tris-buffered saline (pH 7.5) containing 0.2% Tween before the addition of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3000, Bio-Rad, Budapest, Hungary). The antibody–antigen complexes were visualized by means of enhanced chemiluminescence. After scanning, results were quantified by NIH Image J program.

3.7. Localization of AT_1R in the vascular smooth muscle layer

We investigate the pattern of distribution of angiotensin AT_1R on carotid artery sections in Wistar-Kyoto rats. Immunohistochemistry using anti-AT_1 antibodie was performed on perfused-fixed/paraffin-embedded carotid arteries from rats. 3,3'-Diaminobenzidine tetrahydrochloride (DAB; activated by hydrogen peroxide) staining revealed distinct AT_1 labeling of all artery layers from WKY rats,

4. STATISTICAL ANALYSES

Based on the design of the actual experiment, for statistical analyses one-way ANOVA with post hoc test, and Tt-test were used, as appropriate. All results are presented as means ± S.E.M. The level of significance was set at $p<0.05$.

5. RESULTS

5.1. Changes in the mean arterial blood pressure of rats as a function of age

The mean arterial blood pressure of rats increased from newborn until the age of 6 months and then it did not change significantly until the age of 30 months.

5.2. Changes in the vasomotor responses of carotid arteries to the first administration of Ang II as a function of age

The vascular contractions of carotid arteries to the first administration of Ang II significantly increased from newborn (8 days) to the age of 6 months then it decreased to the age of 30 months, indicating the changes in the contractility of smooth muscle.

5.3. Changes in the vasomotor responses of carotid arteries to the second administration of Ang II as a function of age

The second administration of Ang II significantly increased from newborn (8 days) to the age of 2 months then it decreased to the age of 30 months.

5.4. Changes in the difference of vasomotor responses of carotid arteries between second and first administrations of Ang II as a function of age

The magnitude of tachyphylaxis, (calculated the difference between the second and first Ang II-induced contractile responses of carotid arteries) increased from newborn (8 days) to the age of 9 months, then decreased to the age of 30 months.

5.5. Characteristic of vasomotor response curve to Ang II-induced administration

The time to peak increased from newborn (8 days) to adult life (6 months), than decreased to the old age (24 months). The time from peak to baseline decreased from newborn (8 days) to old age (24 months)

5.6. Vasomotor responses of carotid arteries to Ang II in the presence and absence of the endothelium

The potential modulatory role of endothelium was tested in isolated carotid arteries of 2- and 24-month-old rats. We have found no significant differences in the Ang II-induced contractile responses of endothelium-intact and endothelium-denuded carotid arteries.

5.7. Vasomotor responses of carotid arteries to Ang II in the presence and absence of the AT_2R-blokker

The potential modulatory role of AT_2R was tested in isolated carotid arteries of 2- and 24-month-old rats. We have found no significant differences in the Ang II-induced contractile responses of AT_2R in young and old carotid arteries.

5.8. Changes in AT_1R-mRNA level in carotid arteries as a function of age

The relative AT_1R mRNA level substantially changed as a function of age: it significantly increased from newborn (8 days) to the age of 12 months then decreased to the age of 30 months.

5.9. Changes in AT_1R protein density in carotid arteries as a function of age

The AT_1R protein density significantly increased from newborn (8 days) to the age of 16 months, and then it decreased to the age of 30 months.

5.10. Changes in AT_2R mRNA level in carotid arteries as a function of age

The AT_2R mRNA level substantially changed as a function of age: it significantly increased from newborn (8 days) to the age of 18 months then did not change to the age of 30 months.

5.11. Localization of AT_1R in the wall of carotid arteries

The AT_1R-immunocomplexes are present in the dominantly in the smooth muscle layer of carotid arteries.

6. DISCUSSION

In the present thesis, we have focused our investigation on the contractile responses of isolated rat carotid arteries to Ang II as a function of age.

The novel findings of the present thesis are as follows:

(1) MABP changes as a function of age: increases from new born until adult life, than decreases to scenesence age;

(2) The first Ang II-induced contractile responses increased from newborn until adult age, then decreased in senescent rats, exhibiting a “bell-shaped” curve;

(3) The second Ang II-induced contractile responses reduced and showed a similar pattern to that observed at the first administration as a function of age;

(4) The charachteristic of the Ang II-induced vasomotor curves, also show and age dependency

(5) The Ang II-induced an endothelium and AT_2R independent vasomotor response;

(6) Vascular AT_1R mRNA and protein expression showed similar pattern as vasomotor responses to Ang II, first increased, and then decreased as a function of age;

(7) Vascular AT_2R mRNA expression increased from new born to the adult life, did not change as a function of age;

(8) Vascular AT_1R are present dominantly in the smooth muscle layer of carotid arteries.

The important role of the renin - angiotensin system in the regulation of peripheral resistance and thus blood pressure has been well investigated and described previously (Nguyen, 2011). It has been shown that Ang II is one of the key molecules involved in the regulation of cardiovascular system, and it has many other functions (Nguyen, 2011). It is also known that the cardiovascular system is greatly affected by aging (Marin, 1999), among others aging likely affects the regulation of vascular resistance (Diz, 2008), which is known to be greatly influenced by Ang II (Wakabayashi, 1990). Thus, it seemed to be important to elucidate the vasomotor effect of Ang II as a function of age, which may also shed light on its many other functions. Interestingly, little is known regarding the aging-induced changes in the magnitude of vasomotor response elicited by Ang II, which, however, would be important to know because constriction of arterial vessels is the final effector mechanism modulating the resistance of vascular system by Ang II (Wakabayashi, 1990). The present study was conducted in carotid arteries isolated from rats from newborn (8 days) to senescence (30 months), which correspond to humans aged ~80 to 90 years (Franklin, 1997). Thus, our findings are important regarding the role of age-dependent regulation of arterial resistance by Ang II and its mediation by vascular AT_1R. In the present study, we have found that the systemic blood pressure of rats increased from newborn until the age of 4 months, and then it decreased to the age of 30 months. These findings are in agreement with findings of others (Cassis, 2010), showing the similar trends of changes in blood pressure with age in rats (Anisheenko, 2010) and humans (Franklin, 1997).

6.1. Physiological importance

The findings with the first and second administrations show that aging alters the mechanisms responsible for restoring the functional availability of AT_1Rs that can be activated. Although previous studies showed similar increase in the contractile responses of arterial vessels, they did not investigate the responses of very old and senescent rats (24–30 months). The findings that both Ang II-induced vascular contractility and systemic blood pressure are lower in very young and in old and senescent ages compared to the adults suggest different contribution of vascular AT_1Rs in determining vascular resistance and systemic blood pressure. There are a number of possible explanations for the findings that in very old and senescent age, vasomotor response to Ang II declines. For example, aging can lead to the alterations of AT_1Rs, such as changes in their density and/or sensitivity to action of agonist (Chiba, 1986). Although the physiological role of tachyphylaxis is still not clearly defined, nevertheless it gives the opportunity to investigate the behavior and signaling of AT_1R, all of which are greatly affected by aging. This leads to the altering of not only the vasomotor responses to Ang II but also other signaling mechanism attached to these receptors and thus stimulated by Ang II, such as activation of nicotinamide adenine dinucleotide phosphate oxidase and consequent free radical production

(Paravicin, 39) or cellular growth processes during hypertrophy of tissues (Xu, 2010), all of which may have physiological and clinical significances in aging.

7. SUMMARY

In conclusion, the novel findings of the present study are that Ang II-induced vascular contractions, tachyphylaxis, vascular AT_1R mRNA level, and protein expressions substantially change from newborn to senescence, showing bell-shaped curves. Thus, we propose that primarily genetic programs determine the regulation of vasconstrictor responses to Ang II, in part, via the regulation of the functional availability of vascular AT_1Rs. Although further studies are warranted to understand the role of vascular renin-angiotensin system in the regulation of arterial resistance from newborn to senescence age, these findings add important novel aspects to our understanding of the age-dependent regulation of periheral vascular resitance.

ACKNOWLEDGEMENTS

I would like to thank Professor Dr. Akos Koller, my supervisor for tutoring, mentoring and funding this research, from whom I have learned how to do scientific research. I whole heartedly thank to my colleagues and teachers Prof. Janos Hamar, Peter Cseplo MD, Zsolt Springo MD, Ivan Ivic MsC, Robert Matics CsC, Peter Degrell MD, PhD, Prof. Miklos Szekely, Peter Kanizsai MD, PhD, Prof. Lajos Bogar, Erzsebet Ezer MD, Gabor Komaromi MD, Katalin Szenohradszki MD, Klara Nagy MD, Tunde Visnyei, Dalma Dusikne, Emoke Potóné, Eszter Pakai and also to my family for their continuous help and valuable advices.

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Cumulative impact factor: **12.365**

Independent citations: **7**

Number of citable abstracts: **43**

Impact factor of citable abstracts: **138.79**

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