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Bradykinin –induced vasodilatation: Role of age, ACE1-inhibitory peptide, mas- and bradykinin receptors

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ABSTRACT

Bradykinin exerts its vascular actions via two types of receptors, the non-constitutively expressed bradykinin receptor type 1 (BR1) and the constitutive type 2 receptor (BR2). Bradykinin-induced vasorelaxation is age-dependent, a phenomenon related to the varying amounts of BR1 and BR2 in the vasculature.

Isoleucine-proline-proline (Ile-Pro-Pro), a bioactive tripeptide, lowers elevated blood pressure and improves impaired endothelium-dependent vasorelaxation in hypertensive rats. It inhibits angiotensin converting enzyme 1 (ACE1). Other mechanisms of action have also been postulated.

The aims of the study were to clarify the underlying mechanisms of the age-dependency of bradykinin-induced vasodilatation such as the roles of the two bradykinin receptors, the mas-receptor and synergism with Ile-Pro-Pro.

The vascular response studies were conducted using mesenteric artery and aorta rings from normotensive 6 wk. (young) and 22 wk. (old) Wistar rats. Cumulative dosing of acetylcholine, bradykinin and angiotensin(1–7) (Ang(1–7))were tested in phenylephrine-induced vasoconstriction with or without 10 min pre-incubation with antagonists against BR1-, BR2- or mas-receptors, Ang(1–7) or ACE1-inhibitors captopril and Ile-Pro-Pro.

The bradykinin-induced vasorelaxation *in vitro* was age-dependent and it was improved by pre-incubation with Ile-Pro-Pro, especially in old rats with endothelial dysfunction. The mas-receptor antagonist, D-Pro$_7$-Ang(1–7) abolished bradykinin-induced relaxation totally. Interestingly, BR1 and BR2 antagonists only slightly reduced bradykinin-induced vasorelaxation, as an evidence for the involvement of other mechanisms in addition to receptor activation.

In conclusion, bradykinin-induced vasorelaxation was age-dependent and Ile-Pro-Pro improved it. Mas receptor antagonist abolished relaxation while bradykinin receptor antagonist only slightly reduced it, suggesting that bradykinin-induced vasorelaxation is regulated also by other mechanisms than the classical BR1/BR2 pathway.

1. Introduction

Rocha e Silva and co-workers [38] as early as 1949 were the pioneers of bradykinin research. They described that trypsin and some snake venoms were able to break down plasma globulin to produce a blood pressure lowering substance which slowly contracted isolated gut preparations *in vitro* [12,37]. They named the substance bradykinin (bradys = slow). In the early 1970s, the important vasoregulatory enzyme, angiotensin-converting-enzyme (ACE) was discovered, and soon afterwards, pharmacological inhibitors were developed for the treatment of hypertension. ACE acts on a circulating inactive decapeptide, angiotensin I, to release the very potent vasoconstricting octapeptide, angiotensin II. This enzyme also breaks down the vasodilator nonapeptide, bradykinin into inactive fragments. In fact, ACE and kininase II are the same enzyme [8,52]. Thus ACE-inhibitors have dual mechanisms in lowering blood pressure.

Bradykinin mediates its actions via two different receptor types; bradykinin receptor type 1 (BR1) and type 2 (BR2) [37]. BR2 is constitutively expressed in many tissues e.g. vascular endothelium and smooth muscle cells. BR1 is non-constitutive, but it can be induced by inflammation. BR2 mediates most of the vascular functions of bradykinin but BR1 can modulate cardiovascular functions when

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inflammation is present in the endothelium like in hypertension [29].

Bradykinin-induced vasorelaxation is known to be age-dependent [28,43]. In young Wistar (6 wk.), Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) (both 8 wk) *in vitro* evidence of bradykinin-induced vasorelaxation was seen in mesenteric arteries. In 12 week old rats, vasorelaxation was impaired and by the time, when the rats had aged to 18 and 22 months, the response was totally abolished. In these aged animals, bradykinin even constricted the arteries, possibly due to increased production of vasoconstricting prostanoids or decreased expression of the vasodilatory BR2 receptors [21,32]. This age-dependent phenomenon was even more pronounced in SHR [9,28].

Several small bioactive peptides are produced from casein when milk is subjected to fermentation such as isoleucine-proline-proline (Ile-Pro-Pro), leucine-proline-proline (Leu-Pro-Pro) and valine-proline-proline (Val-Pro-Pro) [19]. They all are ACE-inhibitors and demonstrated to reduce elevated blood pressure in rats [16,18,45] and in humans (see, meta-analyses [3,36,46,50]. Furthermore, they partially restored impaired endothelium-dependent vascular relaxation in hypertensive rats [16,18,45]. Although the main mechanism of action of these peptides are postulated to be angiotensin converting enzyme 1 (ACE1) inhibition [25,31,44], this property does not explain all their vasoprotective actions.

Present study was aimed to clarify the mechanisms underlying the age-dependency of bradykinin-induced vasorelaxation, especially the role of bradykinin- and mas-receptors as well as the effects of Ile-Pro-Pro on bradykinin responses, in an *in vitro* experimental protocol.

2. Material and methods

2.1. Animals

Normotensive Wistar rats aged 6 wk. (young) and 22 wk. (old) with free access to tap water and rodent basic feed (Harlan, Rossdorf, Germany) were used. The protocol was approved by the National Animal Experimentation Committee of Finland according to EC Directive 86/609/ECC and the Finnish Experimental Animal Act 62/2006s.

2.2. Vascular response studies

After CO_2 anaesthesia, the animals were decapitated, the mesenteric arteries and the thoracic part of the descending aorta were placed in pre-oxygenated (O2/CO2 95%/5%, Aga, Riihimäki, Finland) ice-cold Krebs buffer (pH 7.4–7.6, composition in mM: NaCl 119.0, $NaHCO_3$ 25.0, glucose 11.1, KCl 4.7, $CaCl_2$ 1.6, KH_2PO_4 1.2, $MgSO_4$ 1.2). Some sections of the aorta were collected in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) and stored at −20 °C for further analysis of the receptor protein levels of BR2 and the Ang(1–7) peptide concentration. Changes in vascular tone and endothelial function were measured in standard organ bath chambers with a computerized system (EMKA Technologies, Paris, France). Endothelium-intact superior mesenteric artery and descending aorta rings (about 3 mm long, diameter of aorta appr. 1.2 mm and mesenteric artery appr. 0.8 mm) without adherent adipose and connective tissue were connected to stainless steel hooks (diameter 0.1 mm for mesenteric arteries and 0.2 mm for aortas) and placed in oxygenated (O_2/CO_2 95%/5%) Krebs buffer in organ bath chambers (10 ml, 37 °C).

The protocol of test series for vascular function measurements was as follows: After one hour of equilibration with 1.5 g baseline tone (passive tension caused by stretching) (resting tension was selected based on earlier studies: [20,27,30]), vessel rings were contracted using 60 mM potassium chloride (KCl) solutions to initiate vascular activity, and subsequently this solution was washed out before the actual experiments. The presence of functional endothelium was tested by using cumulative (1 nM–10 μM) acetylcholine (ACh, Sigma-Aldrich, Munich, Germany) doses after phenylephrine (PE, 1 μM, Sigma-Aldrich) contraction. Bradykinin-induced vascular responses were tested by addition of 1 nM–10 μM cumulative bradykinin acetate salt (Bachem, Weil am Rhein, Germany). Bradykinin type 1 and 2 receptor antagonists (for BR1: 1 μM des-Arg9-Leu8-Bradykinin, (Bachem) for BR2: 0.1 μM Hoe-140, (Tocris, Bristol, UK) and mas-receptor antagonists (A-779, 1 μM and D-Pro_7-Ang(1–7), 1 μM, both from Bachem) were used to study the role of the respective receptors in the bradykinin-induced vasorelaxation. The concentrations were based on observations from our earlier studies [5]. However, we are aware that using only one concentration from each inhibitor, agonist and antagonist is a limitation of the study. This kind of cumulative dosing of acetylcholine and bradykinin has widely been used in vascular relaxation studies *in vitro*. Because bradykinin-induced relaxation responses were less than 50% of the maximum, EC_{50}- values could not be calculated.

Preincubation for 10 min with milk casein-derived ACE-inhibitor Ile-Pro-Pro (1 mM, Bachem) or the pharmacological standard ACE-inhibitor, captopril (10 μM, Sigma-Aldrich) was conducted before contraction and the cumulative bradykinin dosing. The role of nitric oxide (NO) and prostanoids in vasorelaxation was evaluated with a 10 min pre-incubation with the non-specific nitric oxide synthase (NOS) inhibitor L-N^G-nitroarginine methyl ester (L-NAME, 100 μM, Sigma-Aldrich) or with the non-specific cyclooxygenase (COX) inhibitor diclofenac (3 μM, Sigma-Aldrich) before contraction with PE and subsequent cumulative bradykinin dosing. All concentrations were the final concentrations in the cuvette. After every individual test series, the cuvettes were washed three times with Krebs buffer and the vessels were equilibrated for 20–30 min before the next measurements. PE-induced constriction did not alter throughout the measurements of each ring. If ACh-induced endothelium-dependent vasodilatation was too small, the rings were discarded due to distraction of endothelium.

2.3. BR2 receptor protein and Ang(1–7) levels

BR2 receptor protein and Ang(1–7) peptide levels were measured from rat aorta tissue using commercial ELISA kits (BR2: Enzyme-linked immunosorbent assay kit for bradykinin receptor BR2, *Rattus norvegicus,* Cloud-clone Corp. Houston, Texas, USA, Ang(1–7) enzyme-linked immunosorbent assay kit for angiotensin 1–7, *Rattus norvegicus*, Cloud-clone Corp). According to the company Cloud-clone Corp the specificity of antibody against the B2 receptor is reliable and targeted to sequence between amino acids Gln37-Gln320 of the B2 receptors.

The isolation protocol was as follows: A small piece of aorta was cut into small sections using a razor blade and the tissue was homogenized with small beads (procedure: 5500 rotation per minute (rpm), 3 times 20 s + 6500 rpm, twice 15 s, Precellys 24, Bertin Technologies, Montigny le Bretonneux, France) in Elisa buffer (NaCl 136 mM, Na_2HPO_4 8 mM, KCl 2.7 mM, KH_2PO_4 1.46 mM, Tween 20 0.001%) containing a complete protease inhibitor cocktail tablet (Roche, Mannheim, Germany). After homogenization, the samples were stored at 4 °C for 30 min to decrease foaming and centrifuged at 12000*g*, 4 °C for 15 min. The total protein concentration of the supernatant was measured using a Pierce BCA protein assay kit (Thermo Scientific, IL, USA). All samples were diluted using Elisa buffer to the same total protein concentration (0.13 μg/μl) before the BR2 and Ang(1–7) assays were performed.

2.4. RT-qPCR analyses

mRNA isolation protocol was as follow: small piece of frozen aorta was pulverized in liquid nitrogen with mortar and pestel. RNA was isolated using Trizol reagent (InVitrogen, Thermo Fisher Scientific, MA, USA) according manufacturer's instructions. Briefly, pulverized tissue was homogenized in Trizol reagent using Prezellys homogenator (5500 rpm, 3 × 20 s) following centrifugation (12 000*g*, 15 min, 4 °C) with chloroform (Sigma-Aldrich). Bright layer after centrifugation was stored and washed using isopropanol (Thermo Fisher Scientific) and ethanol. In the end of the protocol, formed pellet of mRNA was diluted into nucleic acid free water and total RNA concentration was measured using NanoDrop spectrophotometer (Thermo Fisher Scientific).

One μg of mRNA was converted into complementary DNA (cDNA) using commercial kit (iScript, BioRad, California, USA). cDNA was diluted into 1:5 for final RT-qPCR analyses.

In the final reaction for qPCR diluted samples (2 μl), MasterMix (10 μl, iTaq Universal SYBRGreen Supermix, Bio-Rad), forward (F) and reverse primers (R) (1 μl each) and nucleic acid free water (6 μl) were added in final volume of 20 μl. Primers for ACE1 were F 5′ AGTGGGTGCTGCTCTTCCTA'3 and R 5′ATGGGACACTCCTCTGTTGG'3, for BR1 were F 5′ AGGGTTCGTCATCACTAT'3 and R 5′AGGTAGATTTCCGCTATG'3, for BR2 were F 5′TGAGGAACAACGAGATGAAGAAG'3 and R 5′GGAAACCAACACAGCACAAAGAC'3 (adapted from [40]) and for mas-receptors were F 5′TGTGGGTGGCTTTCGATTT'3 and R 5′ATTAGACCCCCATGCATGTAGAA'3 (adapted from [51]). Three different control genes were used (β-actin, GAPDH and LDHA) and primers for those were β-actin F 5′ AGATCAAGATCATTGCTCCTCCT'3 and R 5′ AAAACGCAGCTCAGTAACAGT'3, GAPDH F 5′GCTGCCTTCTCTTGTGACAA'3 and R 5′ ATCTCGCTCCTGGAAGATGG'3 and LDHA F 5′CATCGTGCACTAAGCGGTCC'3 and R 5′ GCAAGCTCATCAGCCAAGTC'3. Before the final analyses, efficiency of the primer pairs was tested and calculated using dilution series from pooled samples. All primers were ordered from Sigma-Aldrich.

RT-qPCR analyses were done using LightCycler 480 Instrument II (Roche, Espoo, Finland) with protocol of initial in 10 min 95 °C followed 40 cycles of denaturation (15 s 95 °C), annealing (30 s 60 °C except 58 °C for mas-receptor) and elongation (30 s 72 °C). In the end of the protocol melt curve analysis was performed (5 s 95 °C, 1 min 60 °C followed temperature increasing 0.11 °C/s until 95 °C). Results were calculated using the method by Vandesompele et al. [48].

2.5. Statistical analyses

Statistical analyses were done using GraphPad Prism (version 5). Student *t*-test (two tailed) or one-way analysis of variance (ANOVA,) followed by Tukey's multiple comparison test was used to calculate statistical differences. Statistics from the concentration-response curves have been done using all concentrations, if not mentioned otherwise. P-values less than 0.05 were considered statistically significant. All results are presented as means ± SEM, except RT-qPCR results as geometrical mean and expression of individual samples.

3. Results

3.1. Vascular responses

Aorta rings were considered to represent large arteries (basic maintenance vessel) whereas mesenteric artery rings were representatives of smaller resistance arteries, which are more important for blood pressure regulation. The relaxations evoked by cumulative doses of bradykinin and angiotensin(1–7) were tested in both vascular rings in the presence of different agonists, antagonists or inhibitors. Cumulative acetylcholine (ACh) –induced vasorelaxation was used as a standard endothelium –dependent measurement to confirm the functionality of the endothelium.

3.1.1. The role of age and tripeptide Ile-Pro-Pro

The ACh –induced, endothelium-dependent vascular relaxation was impaired in the mesenteric arteries of old rats (Fig. 1A), with maximal achievable relaxation being only approximately 40% *vs.* the almost complete (90%) relaxation obtained in young rats. This difference was not seen in the aortas; in that tissue, the maximum relaxation was less than 50% in both young and older groups (Fig. 1B). Phenylephrine-induced vasoconstriction was similar in the mesenteric arteries and aortas of the young and older rats (Fig. 1D), although the mesenteric arteries of the young rats were more sensitive to potassium chloride (KCl) –induced vasoconstriction ($p < 0.01$) (Fig. 1C).

There was more evident vasorelaxation evoked by bradykinin both in mesenteric arteries and aortas of younger rats compared to that detected in the older rats (mesenteric artery $p < 0.01$, aorta $p < 0.01$) (Fig. 2A). In fact, in the mesenteric arteries of old rats, bradykinin did not cause any relaxation but produced even a slight vasoconstriction; a phenomenon which was more marked in the aortas (Fig. 2B). Bradykinin neither relaxed nor constricted the aortas of young rats, (Fig. 2B).

Pre-incubation with Ile-Pro-Pro (1 mM) or Ang(1–7) (10 μM) for 10 min before PE constriction improved bradykinin–induced relaxation slightly in the aortas of young rats ($p < 0.05$.). (Fig. 3B) This was not seen in mesenteric arteries (Fig. 3A). Unexpectedly, pre-incubation with the classical ACE-inhibitor, captopril decreased relaxation in mesenteric arteries ($p < 0.05$) (Fig. 3A) but had no effect on aortas (Fig. 3B).

In the mesenteric arteries and aortas of older rats, pre-incubation with both ACE-inhibitors (Ile-Pro-Pro and captopril) augmented bradykinin–induced relaxation (mesenteric artery $p < 0.01$, aorta $p < 0.001$) (Fig. 3C and D). Interestingly, Ile-Pro-Pro slightly improved bradykinin relaxation more than captopril at the concentrations used especially in aorta ($p < 0.05$).

3.1.2. The role of bradykinin receptors and mas-receptor

Pre-incubation with the bradykinin receptor blockers i.e. BR1 antagonist des-Arg9-Leu8-bradykinin and bradykinin BR2-receptor antagonist Hoe-140, only slightly prevented bradykinin –induced relaxation in the mesenteric artery rings of young rats (p = ns., max. relaxation in control 38%, B1 antagonist 21% and B2 antagonist 22%) (Fig. 4A). These compounds had no clear effect on aorta preparations (Fig. 4B).

The mas-receptor antagonist A-779 increased the ability of bradykinin to cause relaxation in aorta ($p < 0.05$) (Fig. 4B) but had no effect on mesenteric arteries (Fig. 4A). However, another mas-receptor antagonist D-Pro$_7$-Ang(1–7) abolished relaxation totally in both vessels (mesenteric artery $p < 0.05$, aorta p = ns.) (Fig. 4A and 4B). All antagonists were pre-incubated in the cuvettes for 10 min before phenylephrine constriction and bradykinin-induced relaxation.

3.1.3. The role of NOS and COX enzymes

NOS inhibitor L-NAME abolished the bradykinin-induced relaxation in both vessel preparations (mesenteric artery $p < 0.05$, aorta p = ns.). However, a non-selective COX inhibitor diclofenac decreased relaxation in mesenteric arteries ($p < 0.05$) and slightly improved relaxation in aortas. (Fig. 5A and B)

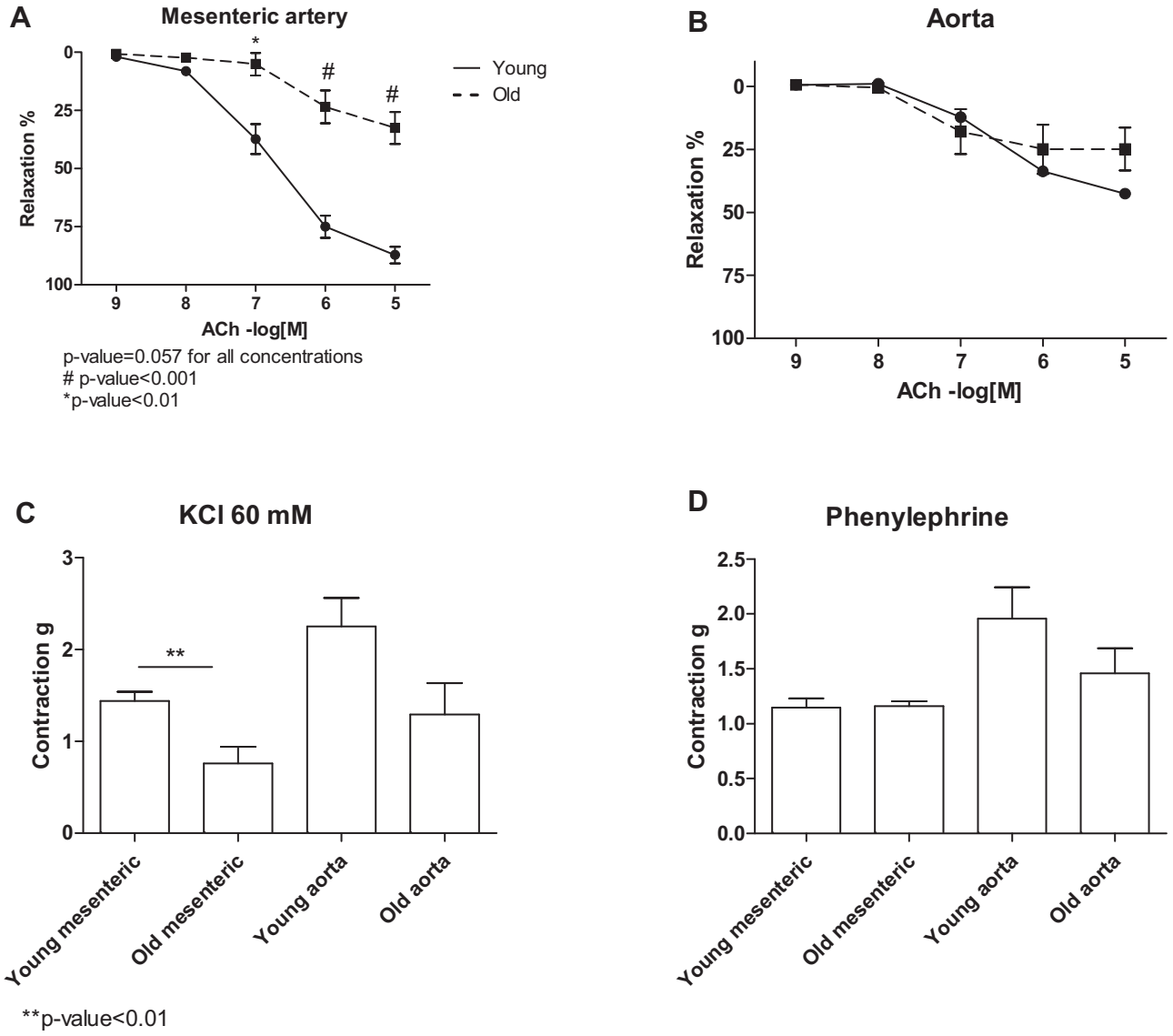

Fig. 1. Acetylcholine (ACh) −induced vasorelaxation (A, B) and phenylephrine (PE) (C) and 60 mM potassium chloride (KCl) (D) −induced vasoconstriction in mesenteric arteries and aortas of young (6 wk.) and old (22 wk.) rats. Data is presented as Mean ± SEM. Mesenteric artery: young n = 16-19 and old n = 7-8. Aorta: young n = 12-15 and old n = 6.

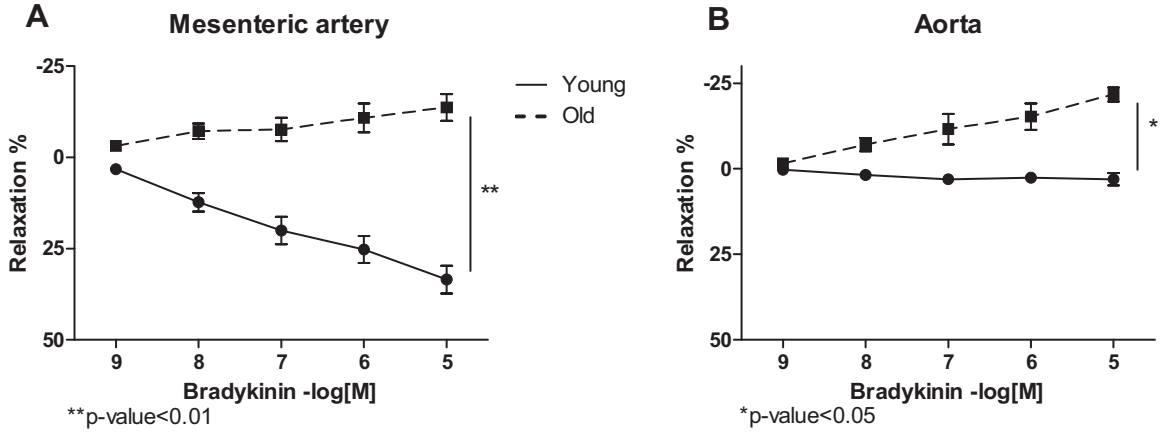

Fig. 2. Bradykinin −induced vasorelaxation in mesenteric arteries (A) and aortas (B) of young (6 wk.) and old (22 wk.) rats. Data is presented as Mean ± SEM. Mesenteric artery: young n = 32 and old n = 8. Aorta: young n = 28 and old n = 6.

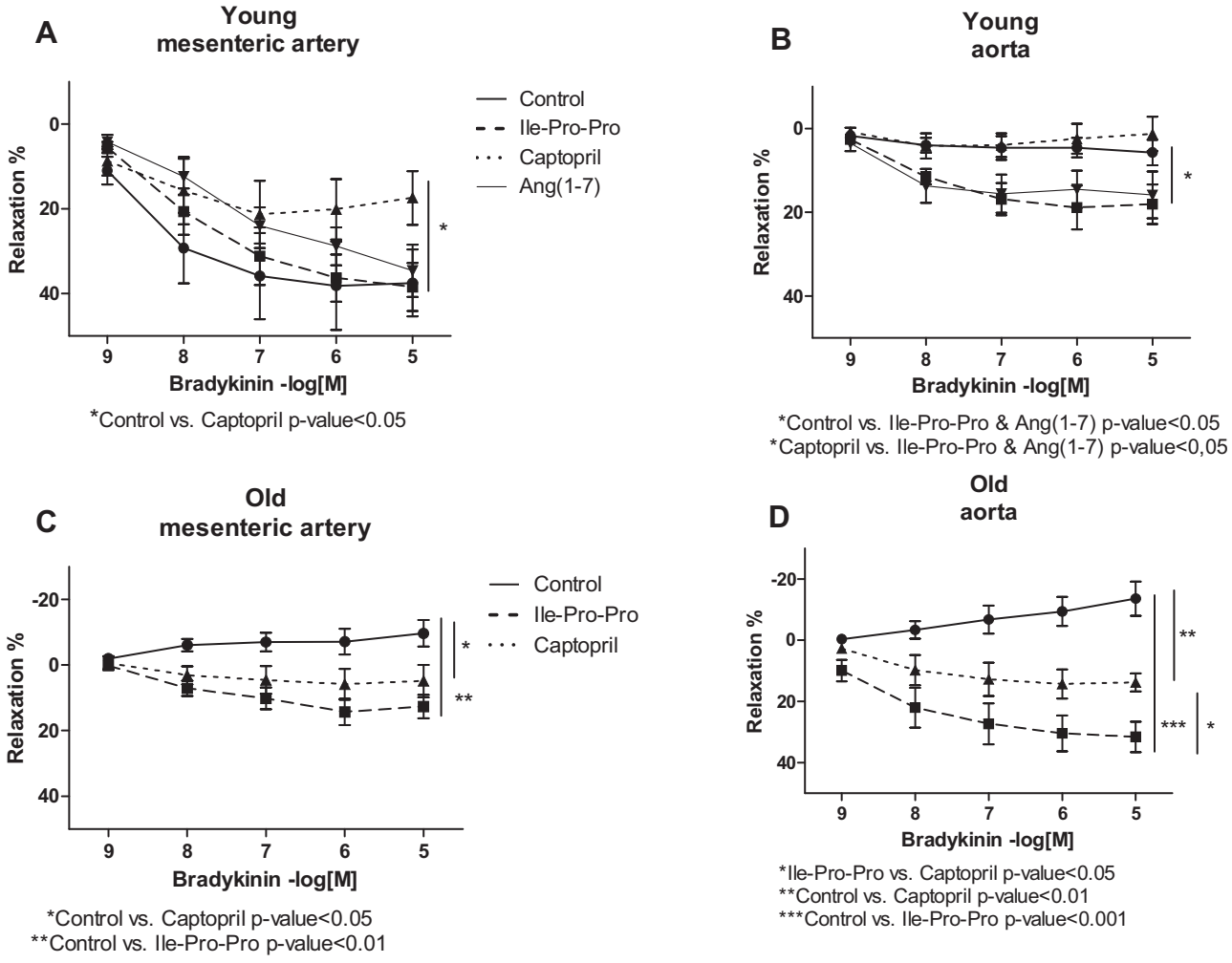

Fig. 3. Effects of 10 min pre-incubation with Ile-Pro-Pro, Ang(1–7) or captopril on bradykinin –induced relaxation of vessels of young (6 wk.) and old (22 wk.) rats. Data is presented as Mean ± SEM. Young mesenteric artery (A): control n = 6, Ile-Pro-Pro n = 8, Ang(1–7) n = 14, Captopril n = 4. Young aorta (B): Control n = 6, Ile-Pro-Pro n = 6, Ang(1–7) n = 12, Captopril n = 6. Old mesenteric artery (C): Control n = 10, Ile-Pro-Pro n = 6321, Captopril n = 4. Old aorta (D): Control n = 6, Ile-Pro-Pro n = 6, Captopril n = 4.

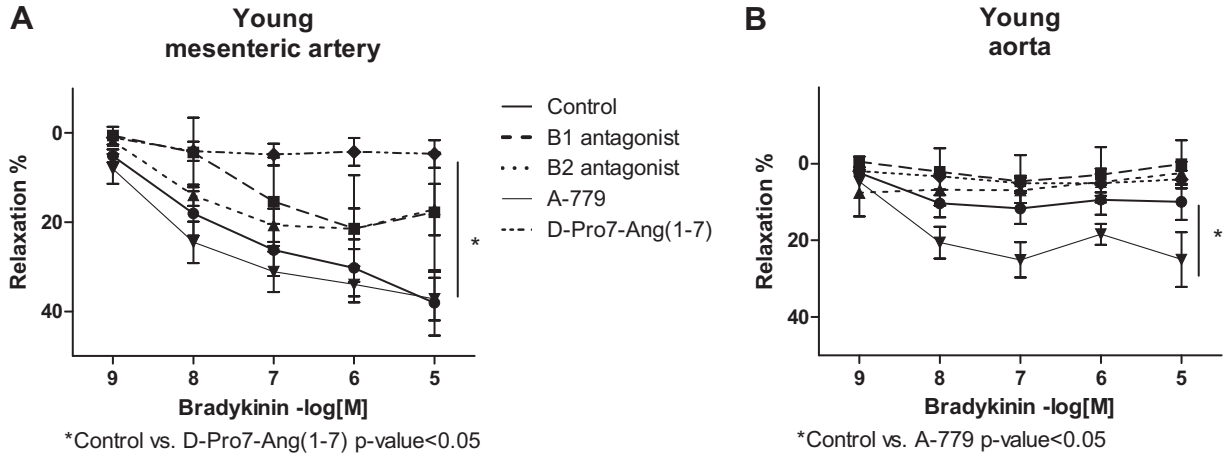

Fig. 4. Effects of the bradykinin receptor BR1 antagonist (des-Arg9-Leu8)-bradykinin (1 μM), BR2 antagonists Hoe-140 (0.1 μM) and mas-receptor antagonist A-779 (1 μM) and D-Pro$_7$-Ang(1–7) (1 μM) on young (6 wk.) rat vessels. Data is presented as Mean ± SEM. Mesenteric artery (A): Control n = 14, BR1 antagonist n = 4, BR2 antagonist n = 8, A-779 n = 10, D-Pro$_7$-Ang(1–7) n = 6. Aorta (B): Control n = 15, BR1 antagonist n = 3, BR2 antagonist n = 6, A-779 n = 6, D-Pro$_7$-Ang(1–7) n = 7.

3.1.4. Ang(1–7)-induced vasorelaxation

Although cumulative doses of Ang(1–7) produced vasorelaxation in both vessels, the extent of the relaxation was more marked in the mesenteric artery (Fig. 6A and 6B). A ten minute pre-incubation with captopril slightly improved the relaxation in aorta ($p < 0.05$) (Fig. 6B). Ile-Pro-Pro slightly improved relaxation in mesenteric arteries in smaller Ang(1–7) concentrations and decreased relaxation on the level of control in higher Ang(1–7)

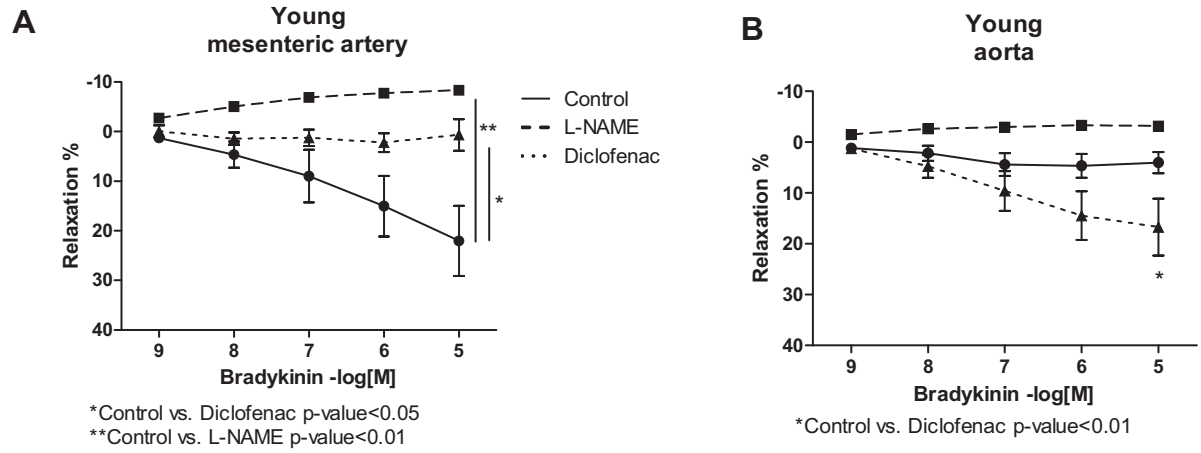

Fig. 5. Effects of the NOS and COX enzymes inhibition using L-NAME (100 μM) and diclofenac (3 μM) on bradykinin-induced vasorelaxation in young (6 wk.) rat. Data is presented as Mean ± SEM. Mesenteric artery (A): Control n = 9, L-NAME n = 4, diclofenac n = 6. Aorta (B): Control n = 12, L-NAME n = 4, diclofenac n = 8.

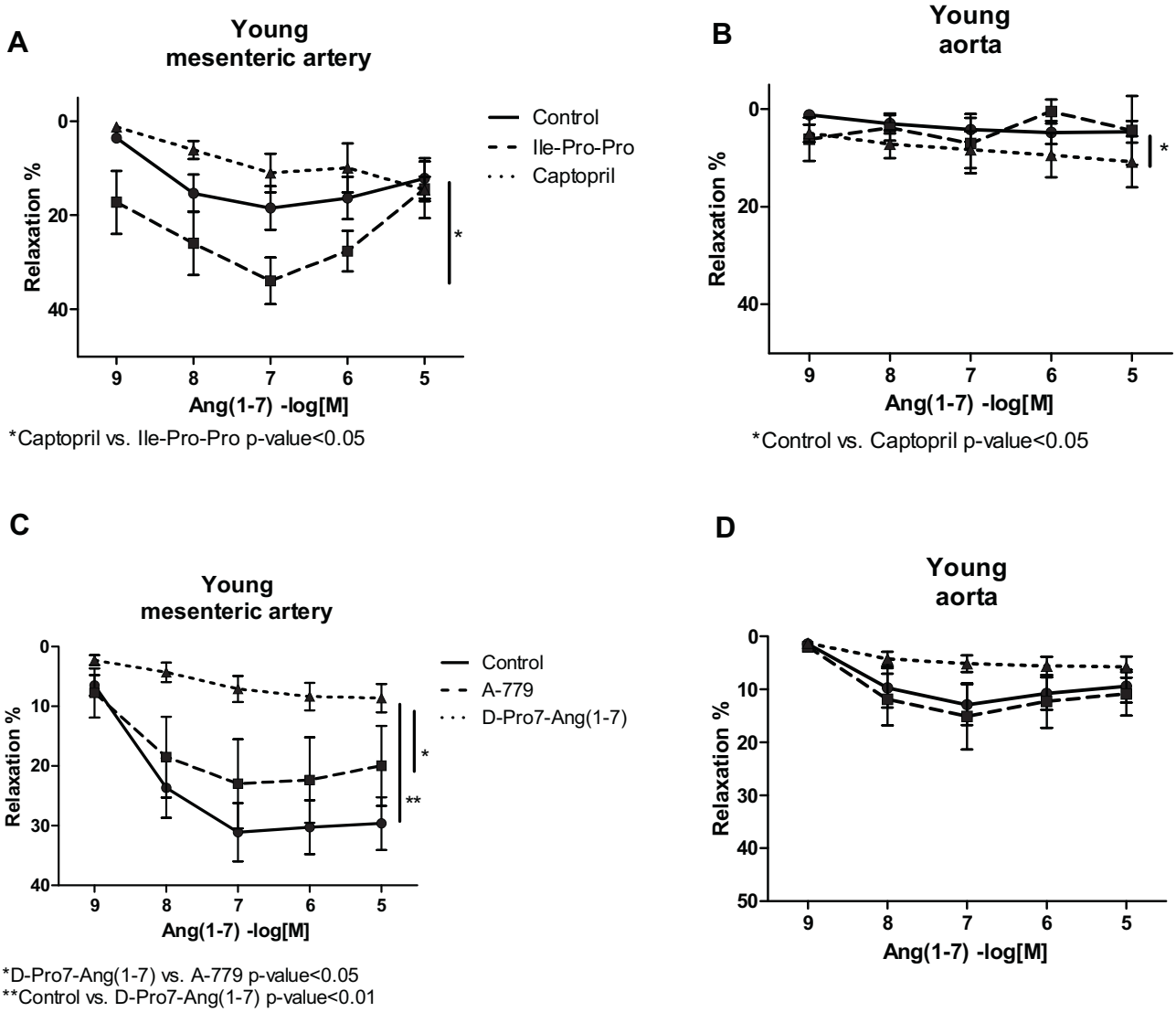

Fig. 6. Angiotensin(1–7) (Ang(1–7)) –induced vasorelaxation in the vessels of young (6 wk.) rats. Preincubation of 10 min with Ile-Pro-Pro, captopril or mas-receptor antagonist A-779 or D-Pro$_7$-Ang(1–7) before constriction and cumulative Ang(1–7) dosing. Data is presented as Mean ± SEM. Mesenteric artery (A): Control n = 13, Ile-Pro-Pro n = 5, Captopril n = 8. Aorta (B): Control n = 13, Ile-Pro-Pro n = 6, Captopril n = 7. Mesenteric artery (C): Control n = 12, A-779 n = 10, D-Pro$_7$-Ang(1–7)n = 5. Aorta (D): Control n = 14, A-779 n = 8, D-Pro$_7$-Ang(1–7) n = 7.

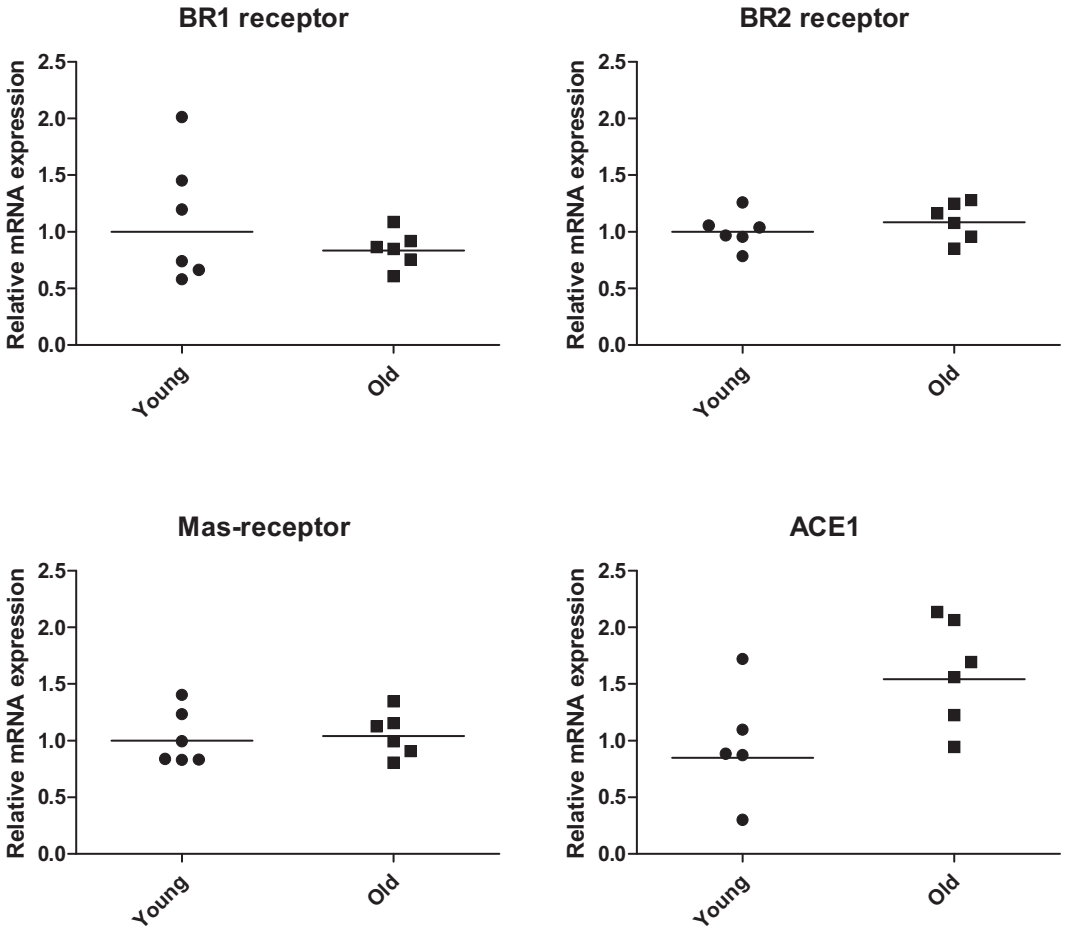

Fig. 7. Relative mRNA expression of BR1 and BR2 receptors, ACE1 and mas-receptors in young and old rat aorta. Results are presented as geometrical mean (line) and expression of individual sample (symbol). N = 5-6 in each group.

Table 1
Levels of bradykinin receptor type 2 protein and angiotensin(1–7) peptide from young (6 wk.) and old (22 wk.) rats aorta. Mean ± SEM values were calculated as a proportion of the total protein values of the samples (pg/μg of total protein). Data is presented as Mean ± SEM. N = 5-6 in each group.

	Young	Old
Bradykinin receptor type 2	130.4 ± 7.9	130.4 ± 11.9
Angiotensin(1–7)	0.156 ± 0.013	0.127 ± 0.013

concentrations (Fig. 6A). It had no effect on aortas (Fig. 6B). Unexpectedly, a pre-incubation with A-779, an antagonist of the mas-receptor (i.e. the receptor for Ang(1–7)), only slightly inhibited Ang(1–7) –induced relaxation in mesenteric arteries (p = ns., max. relaxation in control 31% and A-779 23%) and had no effect on aorta (Fig. 6C and D). However, D-Pro$_7$-Ang(1–7), another mas-receptor antagonist, abolished the relaxation in both vessels (mesenteric artery $p < 0.01$, aorta p = ns.).

3.2. Protein levels

There were no differences in bradykinin receptor BR2 protein and Ang(1–7) peptide levels between young and old rats' aorta (Table 1). Measured values were calculated as a proportion of the total protein values of the samples.

3.3. Gene expression levels

There were no statistical differences in bradykinin receptor BR1 and BR2, ACE1 or mas-receptor mRNA expression levels between young and old rats' aorta (Fig. 7). However, ACE1 gene expression showed to be slightly higher in old rats compared to younger ones ($p = 0.0615$).

4. Discussion

The age-dependency of bradykinin –induced vasodilatation has been postulated to be due to the fact that ageing changes the expression pattern of the bradykinin receptors [21,32]. The density of BR2 is reduced whereas that of BR1 seems to be elevated by ageing that changes the balance towards vasoconstrictory receptors. It has also been suggested that the vasodilator as well as other cardioprotective actions of bradykinin in arteries result from the activation of the BR2 subtype [23,28]. Thus, because the number of BR2 receptors decline during ageing, these actions of bradykinin are abolished and possibly even converted into vasoconstrictive effects, which could evoke ischemic damage in the heart [28]. In general, it seems that with ageing, the levels of kinins actually increase but their response in the target cells reduces [35].

The present study examined the effects of bradykinin on mesenteric artery and aorta rings of young (6 wk.) and old (22 wk.) normotensive rats. We tested several pharmacological antagonists to determine the possible contributions of the two bradykinin receptors as well as the mas-receptor. Bradykinin induced a

vasorelaxation which was age-dependent in both the mesenteric artery and aorta. A weak ACE1-inhibitory peptide, Ile-Ile-Pro, augmented the effects of bradykinin in the same manner as the mas-receptor agonist, Ang(1–7). Interestingly, BR1- and BR2-antagonists only slightly reduced the vasodilatory effects of bradykinin, suggesting that other mechanisms may be involved in this phenomenon in addition to BR receptor activation.

Another part of the classical renin-angiotensin-system (RAS), i.e. the heptapeptide angiotensin (Ang(1–7)) –mas-receptor axis (see [2,41]) which may also play a role in bradykininís vasoprotective actions. Ang(1–7), a break-down product by ACE2, has been demonstrated to potentiate bradykinin-induced vasorelaxation [10,11,33,34,47]. We have previously shown that also a bioactive tripeptide, Ile-Pro-Pro, has synergistic effects on bradykinin-induced vasodilatation in mesenteric artery preparations obtained from SHR [5].

Here we describe that ACh –induced endothelium-dependent relaxation after phenylephrine (PE) constriction was impaired in the mesenteric arteries of older rats, (Fig. 1) probably due to some degree of endothelial dysfunction in the aged smaller arteries. This age-dependent impairment was not seen in the aortas, suggesting that if dysfunction develops at all in these vessels, it only appears later than in the small arteries. However, after PE constriction, the maximal ACh –induced relaxation was only 50% from the baseline in both young and old rats, indicating that the aorta is not the most suitable blood vessel for studies on endothelium function.

It has been proposed that ageing also decreases the extent of vasoconstriction via an endothelium-dependent mechanism. Shipley and Muller-Delp [42] described that the responsiveness of coronary resistance arterioles to potassium chloride in both young and old rats and concluded that the decreased constriction observed in the coronary arteries of old rats was endothelium-dependent. In our study, although the level of non-receptor mediated vasoconstriction to potassium chloride was decreased in the mesenteric arteries of old rats (Fig. 1), there was no evidence of impaired constriction to phenylephrine (receptor mediated). This finding supports the concept that these old rats exhibited some degree of endothelial dysfunction in their mesenteric arteries which may have been reflected also in their smooth muscle function.

Bradykinin induced relaxation in the mesenteric arteries of normotensive young rats, but caused slight vasoconstriction in the aortas of older rats (Fig. 2). Previously, Mantelli et al [28] investigated the effects of bradykinin in mesenteric arteries from spontaneously hypertensive rats. The vasoconstriction in old rats was equal or even stronger than that in normotensive rats. It is believed that vasodilatation is regulated via BR2 and BR1 receptors whereas vasoconstriction is attributable to the release of prostanoids [21,28,32,49]. Endothelial inflammation seen in hypertension [15] and during ageing [6] can influence on various endogenous agents. The rats in our study were normotensive, thus even the old animals may have minor local inflammation in the endothelial lining their blood vessels (see, [6,15]).

BR1 has been shown to have a more evident role in disease states where there is the presence of inflammation [29]. It may be that BR2 receptors are more important in bradykinin-induced actions during ageing in healthy rats. Interestingly, there was no difference in mRNA expression (Fig. 7) or the protein levels (Table 1) of BR2 receptors between the young and old groups, although bradykinin-induced vasorelaxation was impaired in the vessels of old rats. This phenomenon differs from the previous findings by our group [32] and others [21,49]. We found no statistical difference in mRNA expression of mas-receptors and BR1 receptors between the groups. It seems that mas-receptor protein expression decreased during ageing in rats suffering metabolic syndrome [14,39] and cardiac hyperthrophy and myocardial infarctions [4]. Rats in the present study were healthy.

The pre-incubation with two ACE-inhibitors, the tripeptide Ile-Pro-Pro and the standard agent, captopril augmented bradykinin-induced relaxation especially in the vessels of old rats (Fig. 3). Both compounds act as ACE1 inhibitors and thus prevent the degradation of bradykinin into its inactive fragments. However, it seems that Ile-Pro-Pro may possess also other mechanisms of action, especially in the vessels of old rats, because Ile-Pro-Pro was able to improve bradykinin-induced relaxation more than captopril, at the concentrations used.

Interestingly, captopril had no effect on vessels of the young rats, but it increased bradykinin-induced vasodilatation in vessels of old rats (Fig. 3). It has been shown that ACE1 activity increases during ageing in aorta [7,22]. It is possible that activity of the ACE1 does not affect the degradation of bradykinin in vessels of young animals, thus inhibition of ACE1 does not improve bradykinin-induced vasodilatation. In arteries of old rats, ACE1 activity is increased and bradykinin is degraded by this enzyme. Based on that, inhibition of ACE1 by captopril improves bradykinin-induced vasodilatation.

Previous studies by our group and others [5,33,47], have revealed that Ang(1–7) is able to augment bradykinin-induced vasodilatation. However, in the present study Ang(1–7) only slightly increased bradykinin-induced relaxation in the aorta of the young rats and had no effects on respective relaxation in mesenteric arteries (Fig. 3). Ile-Pro-Pro caused at least as good relaxation as Ang(1–7) pre-incubation and then subsequent bradykinin dosing. We hypothesise, that Ile-Pro-Pro acts on bradykinin responses in a similar mechanism as Ang(1–7).

Endothelial nitric oxide (NO) is the main player of endothelium-dependent vasodilatation. In the present study, NOS inhibition totally abolished relaxation in both vessels (Fig. 5). Interestingly, COX enzyme inhibition, which blocks the production of endothelial prostanoid formation, decreased relaxation in mesenteric artery but tended to increase relaxation in aortas of the young rats. It might be, that the balance between constricting and dilating prostanoids differs from vessels to vessels in rats.

The pre-incubation with the bradykinin receptor antagonists also only slightly decreased the ability of bradykinin to relax the mesenteric arteries of young rats (Fig. 4). In the aorta, bradykinin did not induce dilatation and receptor blockade had no effects, confirming earlier studies [28] that the bradykinin-induced vasoconstriction affects prostanoid release. Bradykinin activates phospholipase A2 (PLA2) which releases more arachidonic acid from the cell membranes for prostanoid production [1,17,26]. This phenome seems to be regulated through B2 but not B1 receptors.

Ang(1–7) induced only slight relaxation of the aortas of young rats (Fig. 6), in support of the proposal that the aorta is not the best vessel to undertake endothelium-based studies. The tripeptide Ile-Pro-Pro had no effect on Ang(1–7)-induced relaxation, suggesting that it does not directly affect mas-receptors or alternatively that the receptors were already maximally stimulated by the specific agonist Ang(1–7).

Interestingly, the mas-receptor antagonist, A-779, did not totally inhibit Ang(1–7)-induced relaxation of the vessels of young rats (Fig. 6). This can be explained by postulating that A-779 is only a partial agonist of the mas-receptor at the concentration used (1 μM final concentration). It is also possible that A-779 is degraded by an unknown protease during pre-incubation (10 min) and forms active fragments which then may act as receptor agonists. However, these hypotheses will need to be evaluated in future studies. Other tested mas-receptor antagonist, D-Pro$_7$-Ang(1–7) abolished both Ang(1–7)- and bradykinin-induced relaxation. D-Pro$_7$-Ang(1–7) is also an antagonist for the mas-related G-protein–coupled receptor, member D (MrgD) [24] which is a receptor for alamandine, the latest found compound of RAS. It has been shown that Ang(1–7) is an agonist to MrgD receptors [13] and A-779 had no antagonist effects on it [24]. It is possible that in our study Ang(1–7) caused

vasodilatation also through MrgD receptors which were blocked using D-Pro$_7$-Ang(1–7).

In conclusion, we have demonstrated that bradykinin-induced vasorelaxation is age-dependent and that Ile-Pro-Pro, an ACE1-inhibitor obtained from the dairy protein casein, is able to improve bradykinin's properties in the vessels of aged rats. The mechanism by which the tripeptide augments bradykinin vasodilatation may be similar to that encountered with Ang(1–7). Mas-receptor antagonist, D-Pro$_7$-Ang(1–7) abolished bradykinin and Ang(1–7) induced relaxation totally. However, D-Pro$_7$-Ang(1–7) is also an antagonist for MrgD receptors which may partly regulate vasodilatation caused by these agents.

Conflict of interest

There are no conflicts of interest.

Authors' contributions

AS carried out the experimental work.
AS, RK and HV designed the study plan.
AS analyzed the data.
AS, RK and HV wrote the paper.

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