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Age-related changes in the local intestinal renin-angiotensin system in normotensive and spontaneously hypertensive rats

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Tiivistelmä - Referat - Abstract

Reniini-angiotensiinijärjestelmän (RAS) paikallisia toimintoja on kuvattu monissa kudoksissa. RAS:n fysiologinen kokonaisvaikutus on riippuvainen kahden vastavaikuttavan biokemiallisen akselin suhteellisesta aktiivisuudesta. Klassisen akselin muodostavat angiotensiinikonvertaasi (ACE), angiotensiini II (Ang II) ja tyypin 1 angiotensiini II -reseptori (AT1R). Vaihtoehtoiseen akseliin kuuluvat angiotensiinikonvertaasi 2 [ACE2], angiotensiini 1-7 [Ang (1-7)] sekä Mas-reseptori (MAS). Suoliston paikallinen RAS toimii parakriinisena säätelijänä ja osallistuu tulehduksen säätelyyn. Järjestelmän vaikutus paikalliseen kudokseen riippuu klassisen ja vaihtoehtoisen akselin aktiivisuuksien suhteesta. Klassinen akseli edistää ja vaihtoehtoinen akseli lievittää tulehdusta. Paikallisten reniini-angiotensiinijärjestelmien toiminnan ja akselien välisen suhteen tiedetään muuttuvan ikääntyessä. Asiaa ei kuitenkaan aiemmin ole tutkittu suoliston osalta. Tutkimukseni kartoitti paikallisen RAS:n ilmentymistä nuorten ja vanhojen rottien suolistossa. Tutkin jejunumia ja paksusuolta. Päälöydökseni oli, että vanhempien rottien suolistossa ACE:n ja ACE2:n suhde oli korkeampi, sekä entsyymiaktiivisuudella että proteiinikonsentraatiolla mitattuna. Näyttää siltä, että ikääntymisen myötä rottien suoliston paikallisen reniini-angiotensiinijärjestelmän toiminta painottuu klassiselle ACE-välitteiselle akselille. On mahdollista, että paikallisen RAS:n toiminnan painottuminen ACE-välitteiselle akselille liittyy siihen, että iän myötä suolistossa tapahtuu tulehduksen ja sidekudoksen muodostuksen lisääntymistä.

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AGE-RELATED CHANGES IN THE LOCAL INTESTINAL RENIN-ANGIOTENSIN SYSTEM IN NORMOTENSIVE AND SPONTANEOUSLY HYPERTENSIVE RATS

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Local renin-angiotensin systems (RAS) are found in many tissues. The main physiological effects of RAS are driven by the balance between two pathways: the angiotensin-converting enzyme I - angiotensin II receptor type 1 (ACE1-AT1R) axis and the angiotensin-converting enzyme 2 - Mas-receptor (ACE2-MAS) axis. The local intestinal RAS functions both as a paracrine regulator and as a regulator of inflammation. The expression of local RAS is known to change with age in many tissues, but age-related changes in the intestinal RAS have not been studied comprehensively. The present study characterized age-related changes in two main pathways of local RAS in the jejunum and colon of young and adult rats, in normotensive and hypertensive strains. The main finding was that 33-week-old rats exhibit an increased ratio of ACE1/ACE2 activities and protein quantity ratios compared to young rats. As the relationship of ACE1 and ACE2 mediated pathways drives the total physiological effects of RAS, the results indicate that the function of intestinal RAS changes with age. It is possible that age-related increase in ACE1-AT1R axis introduces more pro-inflammatory and fibrogenic conditions in the intestine.

Key words: *local renin-angiotensin system, angiotensin-converting enzyme, intestine, aging, spontaneously hypertensive rat, angiotensin (1-7), Mas-receptor*

INTRODUCTION

The renin-angiotensin system (RAS) is traditionally considered as an endocrine system, which mainly regulates systemic blood pressure. However, RAS components are found in species without a closed circulatory system (1), indicating that RAS has local, tissue-level functions, *e.g.* regulating fluid and electrolyte balance, local blood perfusion, tissue remodeling, and wound healing (2). Local RAS, for example in the intestine, refers to tissue-bound mechanisms of angiotensin peptide formation, which operate independently and are activated and regulated differently from systemic, circulatory RAS (3-5). Locally produced angiotensin peptides seem to act as paracrine regulators with their functions being tissue-specific.

Most effects of RAS are mediated by a pathway consisting of angiotensin-I-converting enzyme 1 (ACE1), angiotensin II (Ang II) and angiotensin II receptor type 1 (AT1R). This pathway is known as the ACE1-AT1R axis. In addition, RAS has alternative functional axes. Firstly, Ang II can activate the angiotensin II receptor type 2 (AT2R), instead of AT1R. Secondly, angiotensin-I-converting enzyme 2 (ACE2) can form angiotensin (1-7) (Ang (1-7)), an agonist of the Mas-receptor (MAS). This forms a pathway called the ACE2-MAS axis. In fact, AT2R and MAS partly oppose the physiological effects of AT1R. Thirdly, renin and prorenin can activate prorenin receptors (PRR), leading to increase in inflammatory cytokines and collagen synthesis (6). The physiological effects of RAS are mainly driven by the balance between the ACE1 – AT1R and the ACE2 – MAS axes (7, 8). Chronic disorders in this balance have been implicated not only in hypertension but also in age-related degeneration and pathogenesis (9, 10).

Expression of angiotensinogen (Agt), renin, ACE1, ACE2, AT1R and AT2R, and MAS have previously been reported in rodent intestine (11-17). Virtually all tissues have the ability to produce the components of RAS (2, 18), but especially the expression of ACE2-MAS axis is high in rat intestine. Gastrointestinal (GI) RAS has a role in the regulation of intestinal inflammation, apoptosis, fibrosis and mucosal protection, with disturbances in this system implicated in ischemia-induced mucosal damage, pathogenesis of inflammatory bowel diseases, and carcinogenesis (2, 7, 19, 20). Especially, ACE2-MAS axis protects the mucosa and helps its recovery from damage (19, 21). Pathological activation of ACE1-AT1R axis may have opposite effects, as it seems to promote colitis development (20). Previous studies have observed that treatment with ACE1 inhibitors or AT1R blockers alleviates chemically-induced colitis in mice (22-27). GI RAS also regulates physiological functions of the intestine, such as electrolyte homeostasis, regional blood perfusion, peptide digestion and transport, absorption of glucose, sodium and water, motility of GI tract, and mucosal secretion (2, 3, 7, 17, 28).

The GI RAS is of particular interest, as distinct RAS components also have additional, RAS-independent functions in the GI tract. ACE1 activity, quantity, and gene expression within the intestine are highest in the brush border of jejunum (13, 16, 29). Two forms of intestinal ACE1 have been identified: membrane bound ACE1 and secretory ACE1 (30). It is likely that intestinal ACE1-like peptides have also digestive properties,

and may participate in the degradation of intestinal hormones like cholecystokinin and gastrin (31, 32). In rats, highest expression of ACE2 is found in small intestine (14). Intestinal ACE2 regulates amino acid transport and local immunity (33, 34). Intestinal Ang II regulates inflammation by stimulating glucocorticoid formation in mice (35), and Ang (1-7) protects the gastric mucosa by decreasing inflammatory cytokines (7).

The digestive functionality of the intestine changes with age. Aging humans and murine models of aging exhibit intestinal epithelial dysfunction, increased prevalence of inflammatory bowel diseases, as well as susceptibility to intestine-derived systemic inflammation. In addition, shrinkage of mucus thickness, decreased GI motility, and changes in absorption are associated with aging (36-41). RAS components might play a role in age-related deleterious effects in the gut. Relationship between local RAS and age appears to be twofold: physiological aging process changes local RAS expression and, on the other hand, age-related changes in local RAS might cause or worsen aging-related degenerative changes. In rodents, age-related changes in local RAS expression have been reported in lungs, in skeleton and the vasculature (9, 42-45). In humans, age-related changes in RAS activation have been associated with the degeneration especially in the kidney and cardiovascular system (46-48). In contrast, age-related changes in the intestinal RAS have not been well characterized in either humans or murine models. Previously, Garrido-Gil *et al.* reported that 18 – 20 month-old rats display higher colonic AT1R gene expression but lower AT2R expression than 2 – 3 month-old rats (49). Additionally, we have shown elevated intestinal ACE1 expression and activity in 26-week old rats, compared to 9-week old rats (50). However, aside from these two studies, a comprehensive picture of the age-related changes in intestinal RAS has been lacking.

The aim of the present study was to characterize age-related changes in intestinal RAS expression and activity. Our focus was especially on measuring activities and protein quantities of ACE1 and ACE2 and comparing the balance of these enzymes, as well as assaying gene expression of the most important RAS components. Assays were made in specimens from both normotensive Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR).

We hypothesized that age-related changes in intestinal RAS would be similar to changes in the lungs and kidney in rats. Therefore, we expected adult rats to show increased intestinal RAS expression, higher activity of ACE1-AT1R axis and downgraded ACE2-MAS axis. As far as we are aware, the present study is the first to comprehensively map changes in the intestinal expression of the two main RAS axes.

MATERIALS AND METHODS

Animals

A total of 18 WKY and 16 SHR were purchased from Janvier Labs (Saint-Benhevin, France). At this point, half of the rats in each strain were 3 weeks (young) and half were 32 weeks (adult) old. The rats were housed in individually-ventilated cages, two animals per cage, in specific pathogen-free laboratory conditions with 12-h light/dark cycle and relative humidity of 55%. The animals had free access to standard rat feed (Harlan, Rossdorf, Germany) and tap water.

At the beginning of the experiment, the rats were weighted and their systolic, diastolic and mean arterial (MAP) pressures were measured using non-invasive tail-cuff method (CODA, Kent scientific corporation, Torrington, CT, USA). Before the measurements, the animals were kept for 10 min at 32ºC in order to make tail artery pulsation more readily detectable. At least three individual measurements were averaged.

After the one-week accommodation period, the animals were anesthetized with isoflurane (5%, Vetflurane®, Virbac, Carros, France) and sacrificed with cardiac puncture and disconnecting of the aorta. Tissue samples from distal jejunum and proximal colon were collected and rinsed with standard phosphate-buffered saline. Samples were stored at –80ºC for later analysis. In the final analysis, 6 – 9 jejunal and 4 colonic samples from each group were used.

The study was approved by National Animal Experimentation Committee of Finland (ESAVI/10598/04.10.07/2017) according to EC Directive 86/609/ECC and Finnish Experimental Animal Act 62/2006.

Western blot

ACE1 and ACE2 protein quantities were analyzed with Western Blot. Tissue samples were homogenized in ELISA buffer (136 mM NaCl, 8 mM Na2HPO4, 2.7 mM KCl, 4.46 mM KH_2PO_4, 0.001% Tween, pH 7.4) using a Precellys 24 homogenizer (Bertin Technologies, Montigny le Bretonneux, France) 3 × 20 s at 5500 rpm at 4ºC. The homogenates were sonicated for 10 s at 25% of the maximal power (VC 505 Ultrasonic Processor, Sonics, Newtown, CT, USA) and centrifuged for 15 min at 13300 g at 4ºC. A commercial kit (Pierce™ BCA Protein Assay Kit, Thermo Fischer Scientific, Waltham, MA, USA) was used to assay the total protein concentration from the supernatants. The supernatants were diluted to the same protein concentration using ELISA buffer and Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) with 5% 2-mercaptoethanol. The proteins were denatured on a heat block at 95ºC for 5 min.

Samples containing 30 µg total protein were loaded in a commercial 4 – 20% Mini-PROTEAN® TGX™ Precast gel (Bio-Rad), including samples from all groups in each gel. After the SDS-Page run, the proteins were transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked with a commercial buffer (Odyssey blocking buffer (TBS), LI-COR, Lincoln, NE, USA) and incubated in a primary antibody solution overnight at 4ºC: ACE1 1:50 (Thermo Fisher), ACE2 1:100 (Santa Cruz Biotechnology, Dallas, TX, USA), β-actin 1:300 (Cell Signaling Technology, Danvers, MA, USA). After washing, the membranes were incubated in fluorescence-labeled secondary antibody solution: ACE1 1:10000, ACE2 1:10000, β-actin 1:20000 (IRDye680LT goat anti-mouse, LI-COR) for one hour at room temperature. The bands were detected by the Odyssey CLx infrared Imaging system (LI-COR) and analyzed by the Image Studio program (LI-COR). The protein quantities were normalized to the quantity of loading control β-actin.

Angiotensin-converting enzyme I and II activity assays

In the measurements of enzyme activity, tissue samples were homogenized in buffer (100 mM Tris - 150 mM NaCl - 1% Triton X-100, pH 8.17) and centrifuged as described above. ACE1 activity was assayed in the supernatants utilizing hydrolysis of synthetic ACE1-specific substrate hippuryl-L-histidyl-L-leucine (HHL; Sigma-Aldrich, St- Louis, MO, USA) to histidyl-leucine (HL). The assay was performed as described by Schwager *et al.* (51). HHL in potassium phosphate buffer was pipetted to a plate, the plate was incubated for 15 min and samples were added. The plate was again incubated for 30 min, after which the reaction was stopped using NaOH. O-phthaldialdehyde was added and the plate was incubated for another 10 min. Solutions were neutralized by adding HCl to the wells and fluorescence was measured at excitation 360 nm and emission 485 nm using Wallac Victor^2 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA). HL standards were used to calculate amount of formed HL in sample wells.

ACE2 activity was assayed using a commercial kit (SensoLyte® 390 ACE2 Activity Assay Kit, Anaspec, Fremont, CA, USA) according to the manufacturer's instructions. Samples were pipetted to a plate and ACE2 substrate solution was added. The plate was incubated at room temperature for 30 min and the reaction was stopped with stop solution. Fluorescence was measured at excitation 330 nm and emission 390 nm using Wallac Victor2 1420 Multilabel Counter. Fluorescence reference standards were used to calculate amount of formed end product in sample wells. ACE1 and ACE2 enzyme activities are presented as an amount of formed end product in one minute, normalized to the total protein concentration of samples (pmol/min/g).

Reverse transcription quantitative polymerase chain reaction

Gene expression was assayed using reverse transcription quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from tissue samples (NucleoSpin RNA Kit, Macherey Nagel, Duren, Germany), analyzed with NanoDrop 2000 Spectrophotometer (Thermo Fisher) and subsequently reverse transcribed to complementary DNA using a commercial kit (iScript™ cDNA Synthesis Kit, BioRad, Hercules, CA, USA), according to the manufacturer's instructions. Primers were designed with Primer-BLAST (NCBI, Bethesda, MD, USA) and Oligo Analyzer version 3.1 (IDT, Coralville, IA, USA). Unless referenced to a previous study (*Table 1*), the primers were custom made by Sigma-Aldrich (St-Louis, MO, USA). Designed primers were validated using control samples with known amounts of target gene expression.

RT-qPCR was run using LightCycler® 480 SYBR Green I Master (Roche Diagnostics Corp., Indianapolis, IN, USA). Drive consisted of initial phase (10 min at 95ºC), followed by 40 cycles of denaturation (15 s, 95ºC), annealing (30 s, 60ºC) and elongation (30 s, 72ºC). Melt curve analysis was performed at the end of experiment.

RT-qPCR results were calculated as normalized relative quantities (NRQ) of messenger RNA (mRNA), as described by Vandesompele *et al.* (52, 53). Three control genes were used: β-actin (*Actb*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and lactate dehydrogenase A (*Ldha*). The control genes were validated previously.

Statistical analysis

Statistical analyses were conducted with SPSS Statistics version 25 (IBM, Armonk, NY, USA). Figures were created with

Table 1. RT-qPCR primer sequences.

Gene	Forward primer 5'->3'	Reverse primer 5'->3'	Ref.
Actb	AGATCAAGATCATTGCTCCTCCT	AAAACGCAGCTCAGTAACAGT	(52)
Gapdh	GCTGCCTTCTCTTGTGACAA	ATCTCGCTCCTGGAAGATGG	(52)
Ldha	CATCGTGCATAAGCGGTCC	GCAAGCTCATCAGCCAAGTC	(52)
Agt	GCAAATCAGTGCCTTCACCC	AAACAAACCCTCACCCCAGGAG	(58)
Renin	CCTCTCTGGGCACTCTTGTT	TGAGCAAGATTCGTCCAAAGC	
Ace1	AGTGGGTGCTGCTCTTCCTA	ATGGGACACTCCTCTGTTGG	(52)
Ace2	GGAGAATGCCCAAAAGATGA	CGTCCAATCCTGGTTCAAGT	(17)
Agtr1	CTCTGCCACATTCCCTGAGTTA	CACTTTCTGGGAGGGTTGTGT	
Agtr2	AGAACAGAATTACCCGTGACCA	TGGAAGGGAAGCCAGCAAAT	
Mas	TGTGGGTGGCTTTCGATTT	ATTAGACCCCATGCATGTAGAA	(59)
Atp6ap2	TTTCGTGTGGCTCATCTCCG	AAAGCACTCGACACCAGAGAA	

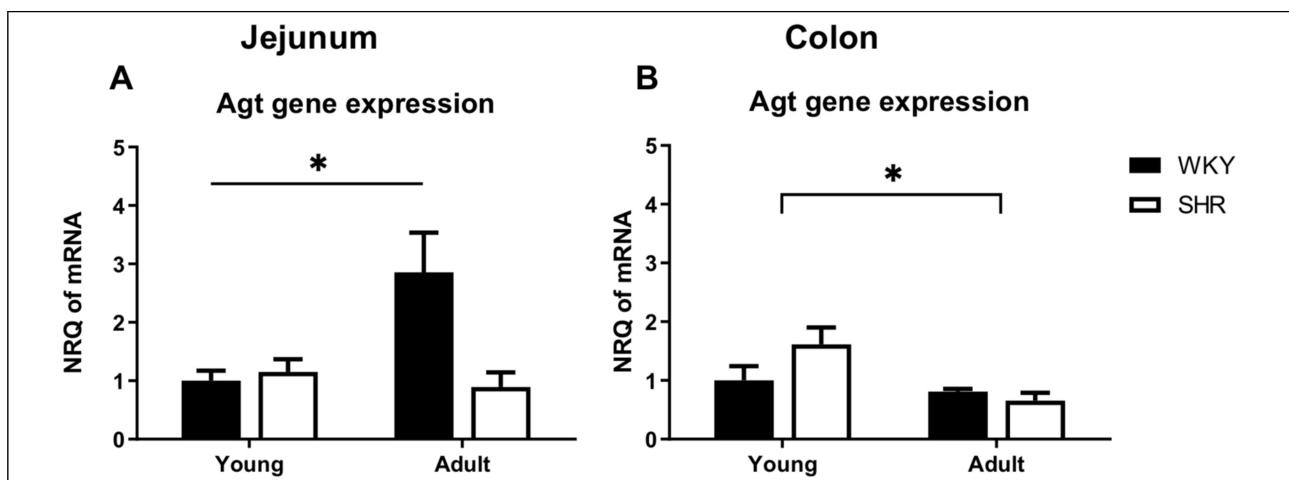

Fig. 1. Angiotensinogen (Agt) gene expression in young (4-week-old) and adult (33-week-old) WKY and SHR. (A): Agt mRNA in jejunum (n = 6 – 9). (B): Agt mRNA in colon (n = 4). The values are normalized to the mean value of young WKY *i.e.* 1.00. Groups are compared with two-way ANOVA. A hooked line marks a difference between the young and the adult rats; a bare line marks a difference between the two indicated groups. * $P < 0.05$.

GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Outliers were analyzed with Tukey's fences, using k-value 2.2 (54). Testing for normality of distribution was done using the Shapiro-Wilk test.

A two-way ANOVA was conducted to analyze differences between the groups. If no statistical interaction between age and strain was observed, the young rats were compared to the adult rats. If significant interaction was present, age-wise comparisons were made separately for the WKY and the SHR strain. Differences between the strains were analyzed similarly. P-values lower than 0.05 were considered statistically significant.

The young WKY group was assigned a mean value 1.00 in each tissue, and the results were normalized accordingly.

RESULTS

Weight and blood pressure

No difference in body weights of the rats between the strains was observed. Young WKY weighted 167 ± 15 g and young SHR 152 ± 9 g (P = 0.41), whereas adult WKY weighted 493 ± 10 g and

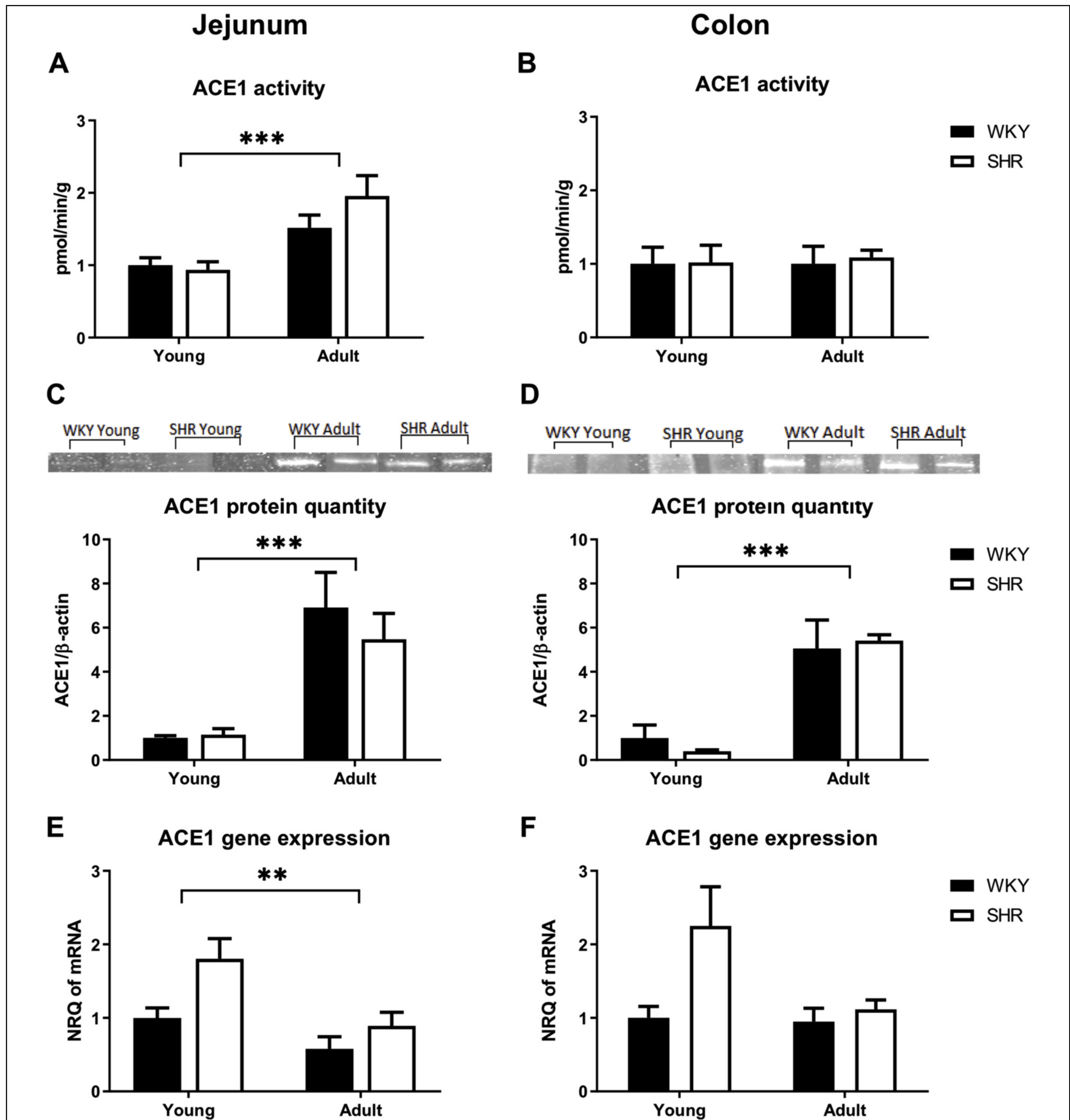

Fig. 2. Angiotensin-I-converting enzyme 1 (ACE1) activity, quantity and gene expression in young (4-week-old) and adult (33-week-old) WKY and SHR. (A): ACE1 activity in jejunum (n = 6 – 8). (B): ACE1 activity in colon (n = 4). (C): ACE1 quantity in jejunum (n = 4). (D): ACE1 quantity in colon (n = 4). (E): Ace1 mRNA in jejunum (n = 5 – 8). (F): Ace1 mRNA in colon (n = 4). The values are normalized to the mean value of young WKY *i.e.* 1.00. Groups are compared with two-way ANOVA. A hooked line marks a difference between the young and the adult rats. ** P < 0.01, *** P < 0.001.

adult SHR 477 ± 19 g (P = 0.49). Adult SHR showed significantly elevated MAP compared to other groups i.e. 152 ± 4 mmHg (P = 0.05). The blood pressures of young SHR (115 ± 5 mmHg), of young WKY (120 ± 6 mmHg) and of adult WKY (112 ± 10 mmHg) were at the same level (P = 0.75).

Angiotensinogen

Jejunal *Agt* gene expression showed a statistical interaction between age and strain; F(1,27) = 7.05; P = 0.013. Adult WKY exhibited higher jejunal *Agt* gene expression than young WKY; 2.86 ± 0.68 versus 1.00 ± 0.17; F(1,15) = 7.85; P = 0.013 (*Fig. 1A*). Adult SHR showed lower jejunal *Agt* gene expression compared to adult WKY; 0.89 ± 0.25 versus 2.86 ± 0.68; F(1,12) = 5.73; P =0.034. In colon, adult rats exhibited lower *Agt* gene expression than young rats; 0.73 ± 0.07 versus 1.31 ± 0.21; F(1,12) = 8.19; P = 0.14 (*Fig. 1B*).

Renin-prorenin receptors and angiotensin-converting enzyme I – angiotensin II receptor type 2 axes

No difference in PRR gene expression between age groups was observed (data not shown). Gene expression of renin and AT2R were too low in both tissues to be quantified accurately.

Angiotensin-converting enzyme – angiotensin II receptor type 1 axis

Jejunal ACE1 activity was higher in adult rats compared to young rats; 1.70 ± 0.16 versus 0.97 ± 0.07; F(1,24) = 19.11; P < 0.001 (*Fig. 2A*). There was no significant difference in colonic ACE1 activity (*Fig. 2B*). ACE1 protein quantity was likewise higher in adult rats than in young, both in jejunum (6.19 ± 0.96 versus 1.07 ± 0.14; F(1,12) = 26.14; P < 0.001; *Fig. 2C*) and in colon (5.23 ± 0.62 versus 0.70 ± 0.30; F(1,12) = 38,92; P < 0.001; *Fig. 2D*).

Jejunal *Ace1* gene expression was lower in adult rats than in the young; 0.71 ± 0.13 versus 1.49 ± 0.21; F(1,21) = 8.67; P = 0.008 (*Fig. 2E*). There was no significant difference in colonic Ace1 expression between the age groups (*Fig. 2F*). *Ace1* gene expression was higher in SHR compared to WKY, both in jejunum (1.35 ± 0.16 versus 0.79 ± 0.16; F(1,21) = 6.1; P = 0.022) and in colon (1.68 versus 0.97; F(1,12) = 5.62; P = 0.035).

Agtr1 gene expression showed a statistical interaction between age and strain, both in jejunum F(1,22) = 7.73; P = 0.011 and in colon F(1,12) = 7.44; P = 0.018). Jejunal *Agtr1* gene expression was higher in adult WKY than in young WKY (3.32 ± 0.87 versus 1.00 ± 0.26; F(1,12) = 6.51; P = 0.025; *Fig. 3A*), whereas SHR showed no age-related difference. Moreover, jejunal *Agtr1* gene expression was lower in adult SHR compared to adult WKY; 0.39 ± 0.03 versus 3.32 ± 0.87; F(1,9) = 6.19; P = 0.035. Colonic *Agtr1* gene expression was lower in adult SHR compared to young SHR; 0.67 ± 0.14 versus 2.79 ± 0.69; F(1,6) = 9.11; P = 0.023 (*Fig. 3B*). Additionally, colonic *Agtr1* gene expression of young SHR was higher compared to young WKY; 2.79 ± 0.69 versus 1.00 ± 0.19; F(1,6) = 6.28; P = 0.046.

Angiotensin-converting enzyme 2 – Mas receptor-axis

Adult rats exhibited lower ACE2 activity, both in jejunum (0.76 ± 0.09 versus 1.10 ± 0.09; F(1,18) = 6.54; P = 0.020; *Fig. 4A*) and in colon (0.39 ± 0.05 versus 0.86 ± 0.16; F(1,12) = 7.94; P = 0.016; *Fig. 4B*). Similarly, jejunal ACE2 protein quantity was somewhat lower in adult rats (0.69 ± 0.22 versus 0.99 ± 0.23; *Fig. 4C*); however, the difference was not significant (P = 0.39). Colonic ACE2 protein quantity was on the same level between the groups (*Fig. 4D*).

A statistical interaction between age and strain was observed in jejunal *Ace2* gene expression; F(1,27) = 7.46; P = 0.011. Adult SHR showed lower jejunal *Ace2* gene expression compared to young SHR; 1.03 ± 0.07 versus 1.70 ± 0.14; F(1,11) = 11.82; P = 0.006 (*Fig. 4E*), whereas expression in WKY was on the same level. Additionally, young SHR showed higher jejunal *Ace2* gene expression than young WKY; 1.70 ± 0.14 versus 1.00 ± 0.11; P = 0.001. Colonic *Ace2* gene expression appeared also to be lower in adult SHR compared to young SHR (1.51 ± 0.68 versus 3.23 ± 0.97), but the difference was not significant (P = 0.21; *Fig. 4F*).

Jejunal *Mas* gene expression showed a statistical interaction between age and strain; F(1,23) = 5.20; P = 0.032. Adult WKY exhibited higher jejunal Mas gene expression compared to young WKY (4.42 ± 1.35 versus 1.00 ± 0.39; F(1,129) = 5.90; P = 0.032; *Fig. 5A*), whereas in SHR no age-related difference was observed. However, *Mas* gene expression was lower in adult SHR compared to adult WKY;

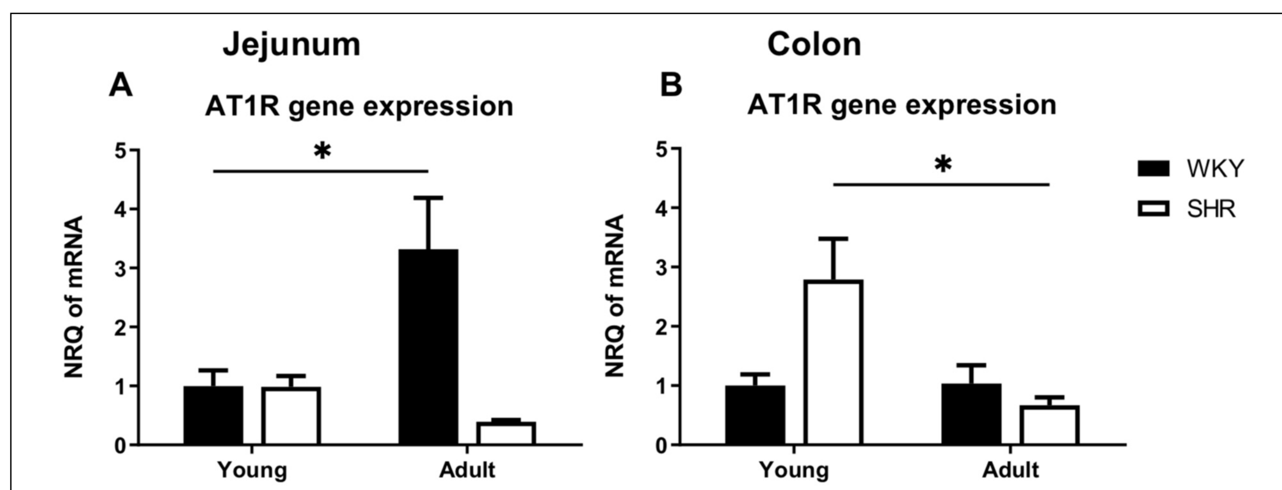

Fig. 3. Angiotensin II receptor type 1 (AT1R) gene expression in young (4-week-old) and adult (33-week-old) WKY and SHR. (A): Agtr1 mRNA in jejunum (n = 6 – 8). (B): Agtr1 mRNA in colon (n = 4). The values are normalized to the mean value of young *i.e.* 1.00. Groups are compared with two-way ANOVA. A bare line marks a difference between the two indicated groups. * P < 0.05.

0.52 ± 0.09 versus 4.42 ± 1.35; P = 0.023. No difference in colonic *Mas* gene expression between the groups was observed (*Fig. 5B*).

Angiotensin-converting enzyme I/angiotensin-converting enzyme II activity and protein quantity ratios

ACE1/ACE2 activity ratio was higher in adult rats compared to young, both in jejunum (2.35 ± 0.22 versus 0.85 ± 0.05; F(1,18) = 39.31; P < 0.001; *Fig. 6A*) and in colon (2.69 ± 0.35 versus 1.15 ± 0.13; F(1,12) = 24.95; P < 0.001; *Fig. 6B*).

Similarly, adult rats showed higher ACE1/ACE2 protein quantity ratios. Jejunal protein quantity ratio showed a statistical interaction between age and strain; F(1,12) = 5.23; P = 0.041. Regardless, jejunal protein quantity ratio was higher in adult rats, both in WKY (15.71 ± 3.81 versus 1.00 ± 0.22; F(1,6) = 14.85; P = 0.008) and in SHR (6.13 ± 1.69 versus 0.96 ± 0.09; F(1,6) = 9.38; P = 0.022; *Fig. 6C*). Colonic ACE1/ACE2 protein

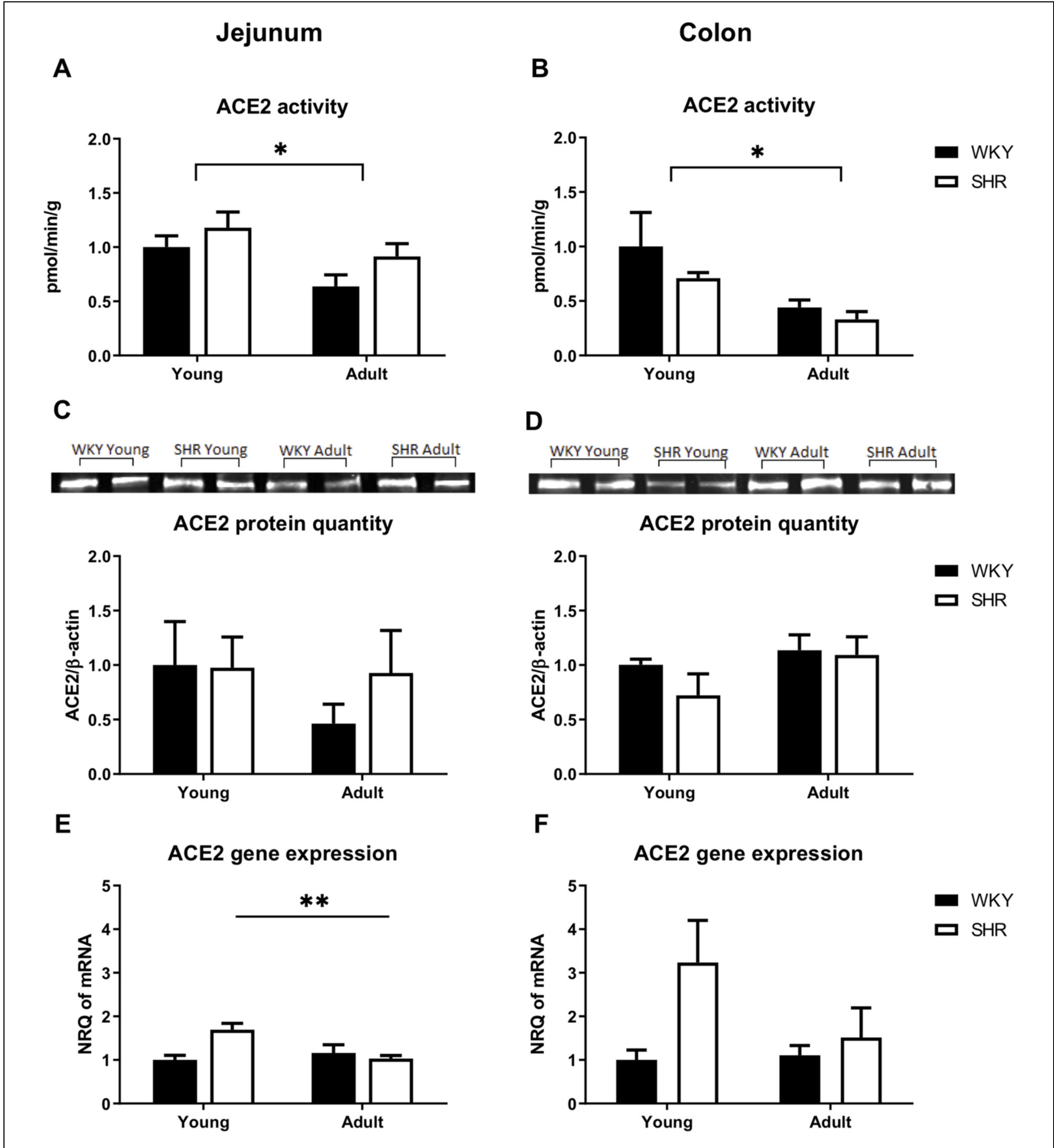

Fig. 4. Angiotensin-I-converting enzyme 2 (ACE2) activity, quantity and gene expression in young (4-week-old) and adult (33-week-old) WKY and SHR. (A): ACE2 activity in jejunum (n = 5 – 6). (B): ACE2 activity in colon (n = 4). (C): ACE2 quantity in jejunum (n = 4). (D): ACE2 quantity in colon (n = 4). (E): Ace2 mRNA in jejunum (n = 5 – 9). (F): Ace2 mRNA in colon (n = 4). The values are normalized to the mean value of young WKY *i.e.* 1.00. Groups are compared with two-way ANOVA. A hooked line marks a difference between the young and the adult rats; a bare line marks a difference between the two indicated groups. * P < 0.05, ** P < 0.01.

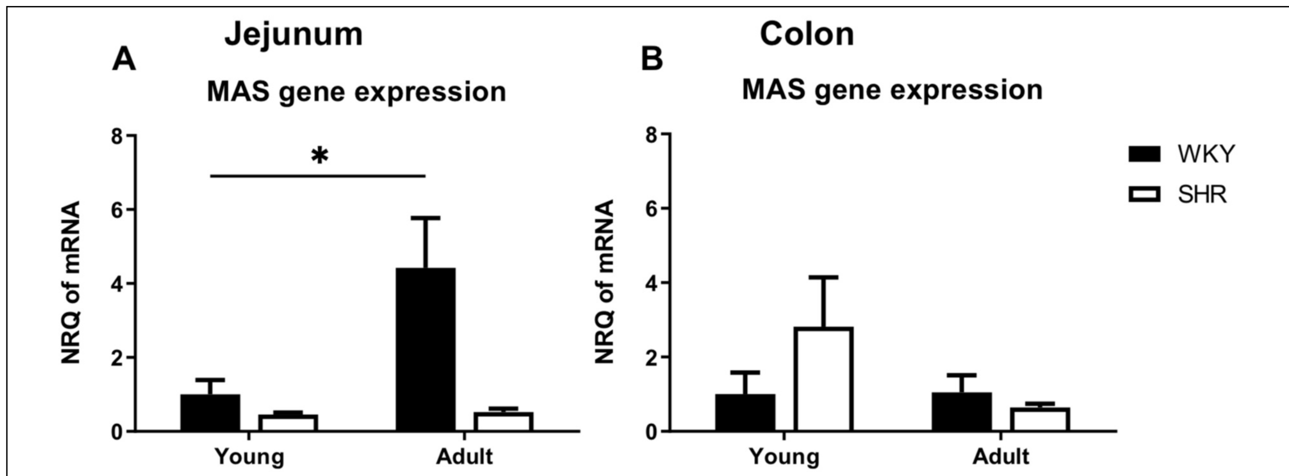

Fig. 5. Mas receptor (MAS) gene expression in young (4-week-old) and adult (33-week-old) WKY and SHR. (A): Mas1 mRNA in WKY jejunum (n = 6 – 7). (B): Mas mRNA in WKY colon (n = 4). The values are normalized to the mean value of young animals *i.e.* 1.00. Groups are compared with two-way ANOVA. A bare line marks a difference between the two indicated groups. * P < 0.05.

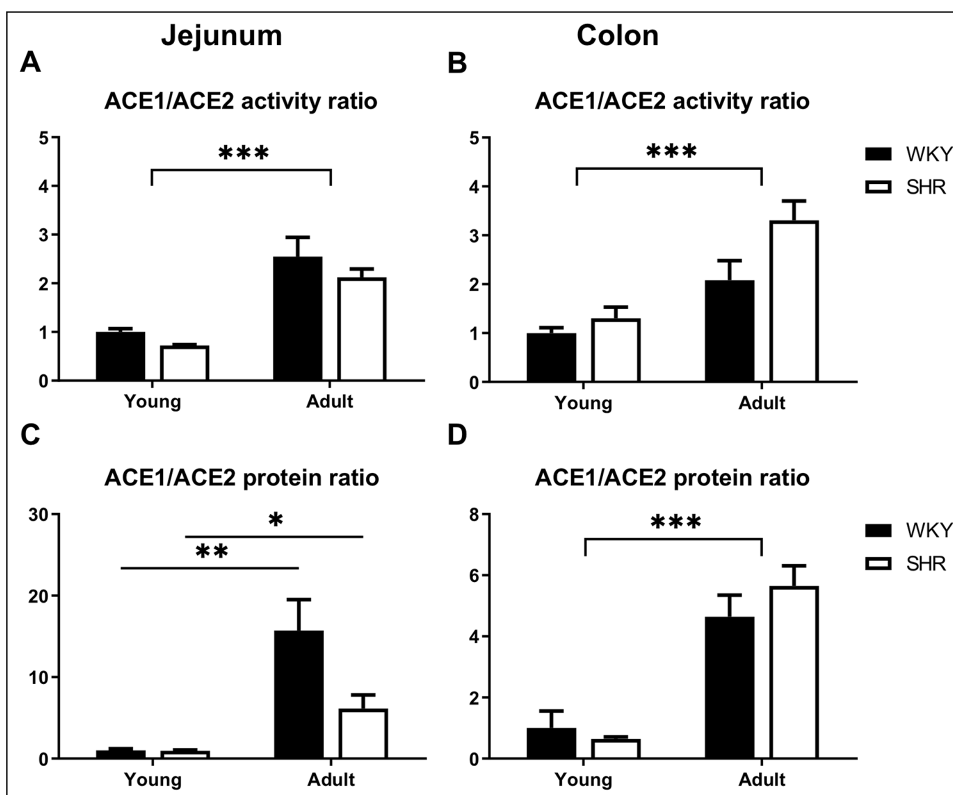

Fig. 6. Angiotensin-I-converting enzyme 1 and 2 (ACE1/ACE2) activity and protein quantity ratios in young (4-week-old) and adult (33-week-old) WKY and SHR. (A): ACE1/ACE2 enzyme activity ratio in jejunum (n = 5 – 6). (B): ACE1/ACE2 enzyme activity ratio in colon (n = 4). (C): ACE1/ACE2 protein quantity ratio in jejunum (n = 4). (D): ACE1/ACE2 protein quantity ratio in colon (n = 4). The values are normalized to the mean value of young WKY *i.e.* 1.00. Groups are compared with two-way ANOVA. A hooked line marks a difference between the young and the adult rats; a bare line marks a difference between the two indicated groups. * P < 0.05, ** P < 0.01, *** P < 0.001.

quantity ratio was likewise higher in the adult rats; 5.15 ± 0.49 versus 0.82 ± 0.27; F(1,12) = 60.07; P < 0.001 (*Fig. 6D*).

DISCUSSION

The presence of local RAS in different parts of the GI tract has been established and its function and regulation are becoming clearer (2, 7, 19). However, age-related changes in the expression and function of intestinal RAS have been poorly known. In the present study, we examined age-related changes in intestinal RAS with prominent age difference, using 4 weeks and 33 weeks old rats. We also examined hypertensive SHR alongside normotensive WKY to investigate whether local RAS in the intestine is altered in hypertensive animals, similar to what is seen in vasculature (47).

ACE1/ACE2 activity and protein quantity ratios were consistently elevated in adult rats, in both jejunum and colon. This supports our hypothesis that, in relative terms, ACE2 activity declines with age, and the balance of intestinal RAS changes towards the ACE1-AT1R axis. This change of balance can have variety of physiological and pathophysiological consequences to age-related changes in the intestine. According to the literature, activation of intestinal ACE1-AT1R axis may cause increased inflammation and fibrotic changes, and consequently make the intestine more vulnerable to ischemia, immune-based diseases and malignancies (2, 19, 20).

Both the activity and the quantity of ACE1 increased with age. It has been previously shown that in rats intestinal ACE1 activity is high at birth but decreases rapidly during the first weeks of postnatal life (55). Our results show that intestinal ACE1 activity rises again as the animals reach 33 weeks of age, supporting our hypothesis that activity in the ACE1-AT1R axis increases with age. Our results are also consistent with a previous study by our group, in which adult (26-week-old) WKY and SHR exhibited a higher jejunal ACE1 activity than young (9-week-old) rats of these strains (50). According to our results, while colonic ACE1 protein quantity was higher in adult rats, no difference in colonic ACE1 activity was observed. This is likely due to low total ACE1 activity in colon and different sensitivity of assays: increased ACE1 quantity did not reflect to assayed ACE1 activity.

Despite adult rats having higher ACE1 activities and elevated ACE1 protein quantities, adult rats exhibited lower jejunal *Ace1* gene expression and no difference in colonic *Ace1* expression. *Ace1* expression was also consistently higher in SHR compared to WKY counterparts, although no differences in ACE1 activity or quantity between the strains was observed. This suggests that ACE1 production is not solely regulated at the level of gene expression. We have observed the same phenomenon in two previous studies. One study, using 4-week and 18-week-old WKY, found that whereas ACE1 activity and protein quantity were markedly higher in aorta and kidney of the adult rats, no differences in *Ace1* expression were detected (56). Another study, using 10-week-old BALB/c mice, found that there was no correlation between assayed colonic ACE1 protein shedding and *Ace1* gene expression (57). In fact, apparent discrepancies between gene expression data and protein quantities are rather common (58).

ACE2 activity decreased with age, both in jejunum and colon. Jejunal protein quantities were also somewhat lowered, however not significantly. Consistent with activity assays, *Ace2* expression was lowered in adult SHR in comparison to young counterparts. These results indicate that decrease in ACE2 activity in old animals is likely to occur both at the level of gene expression and post-translationally. It is also possible that low expression of ACE2 in colon again reduced correlation of colonic activity and protein quantity data.

Agt gene was locally expressed in both jejunum and colon. In WKY, jejunal *Agt* gene expression increased with age. Jejunal *Agt* expression was not increased with age in SHR, and *Agt* expression was lower in adult SHR compared to adult WKY. These results indicate that jejunal *Ang* gene expression is suppressed in adult SHR, possibly due to hypertension. However, as locally expressed *Agt* is considered to be physiologically insignificant under normal conditions (5), one could speculate that intestinal RAS mostly relies on systemically derived substrate.

The *Agtr1* gene expression changed differently with age in WKY and in SHR animals. Jejunal *Agtr1* expression was increased in adult WKY as compared to their young counterparts. This would be consistent with strengthening of the ACE1-AT1R axis with age. In contrast, adult SHR had less colonic *Agtr1* expression than young SHR, and jejunal *Agtr1* expression was lower in adult SHR compared to WKY counterparts. These results suggest that the hypertension developing in adult SHR is a negative feedback regulator of *Agtr1* expression also in the intestine.

Mas gene expression was higher in adult WKY when compared to young WKY. That is to say that adult WKY displayed upregulated gene expression of both main receptors, *Agtr1* and *Mas*, as well as increased local *Agt* gene expression. This would support our hypothesis of increased intestinal RAS expression with age.

Interestingly, expression of the same components, *Agt*, *Agtr1* and *Mas*, were lower in hypertensive adult SHR than in normotensive counterparts. At the same time, young rats from both strains, exhibiting similar blood pressure levels, had similar intestinal RAS expression, differing only in colonic *Agtr1* and jejunal *Ace2* expression. Therefore, it appears that downregulated *Agt, Agtr1* and *Mas* gene expressions in SHR are related to increased blood pressure in adult SHR. It appears that the regulation of intestinal RAS shares certain attributes with the regulation of RAS in vasculature.

Agtr2 gene expression was low and not accurately measurable in either strain. While AT2R has important roles during fetal development, its expression is substantially downgraded after birth in most tissues (59). Some *Agtr2* expression has been detected in the rat intestine, but physiological relevance of intestinal AT2R is not well known (15).

No measurable *renin* expression could be detected in the rat intestine. Although there have been earlier reports of intestinal *renin* expression, these results have not been reliably reproduced (60). It is likely that local *renin* expression is normally negligible and may only increase after some pathological stimulus, such as ischemia (4, 61). The rats exhibited some intestinal PRR gene expression, but no differences were observed between the age groups or the strains.

In summary, as far as we are aware, this is the first study to characterize age-related changes in rat intestine in the two main RAS pathways: ACE1-AT1R axis and ACE2-MAS axis. The main finding of the study was that 33-week-old rats exhibited increased ACE1/ACE2 activity and protein quantity ratios, when compared to young counterparts, in both normotensive and hypertensive strains. As the relationship of ACE1 and ACE2 mediated pathways drives the final physiological effects of RAS, the results indicate that the function of intestinal RAS changes with age. It is possible that age-related increase in ACE1-AT1R axis introduces pro-inflammatory and fibrogenic conditions in the intestine.

H. Salmenkari and R.A. Forsgard equally contributed to this work.

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