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ORIGINAL ARTICLE

Obesity and aging affects skeletal muscle renin–angiotensin system and myosin heavy chain proportions in pre-diabetic Zucker rats

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Abstract

There is a gap in the knowledge regarding regulation of local renin–angiotensin system (RAS) in skeletal muscle during development of obesity and insulin resistance in vivo. This study evaluates the obesity- and age-related changes in the expression of local RAS components. Since RAS affects skeletal muscle remodelling, we also evaluated the muscle fibre type composition, defined by myosin heavy chain (MyHC) mRNAs and protein content. Gene expressions were determined by qPCR and/or Western blot analysis in musculus quadriceps of 3- and 8-month-old male obese Zucker rats and their lean controls. The enzymatic activity of aminopeptidase A (APA) was determined flourometrically. Activation of renin receptor (ReR)/promyelocytic leukaemia zinc finger (PLZF) negative feedback mechanism was observed in obesity. The expression of angiotensinogen and AT1 was downregulated by obesity, while neutral endopeptidase and AT2 expressions were upregulated in obese rats with aging. Skeletal muscle APA activity was decreased by obesity, which negatively correlated with the increased plasma APA activity and plasma cholesterol. The expression of angiotensin-converting enzyme (ACE) positively correlated with MyHC mRNAs characteristic for fast-twitch muscle fibres. The obesity- and age-related alterations in the expression of both classical and alternative RAS components suggest an onset of a new equilibrium between ACE/AngII/AT1 and ACE2/Ang1–7/Mas at lower level accompanied by increased renin/ReR/PLZF activation. Increased APA release from the skeletal muscle in obesity might contribute to increased plasma APA activity. There is a link between reduced ACE expression and altered muscle MyHC proportion in obesity and aging.

Keywords Renin–angiotensin system · Angiotensin II · Age · Obesity · Insulin resistance · Skeletal muscle · Muscle fibre types · Myosin heavy chain

Introduction

The renin–angiotensin system (RAS) is well known as an essential regulator of systemic blood pressure as well as fluid and electrolyte homeostasis. Angiotensin II (Ang II) has long been considered the single critical product of the RAS. Ang II acts via two types of receptors: AT_1 and AT_2. The most of Ang II effects are mediated by the AT_1 receptor, including vasoconstriction, hypertrophy and cellular growth [3]. Previous studies show that the AT_1-mediated Ang II signalling is linked to production of reactive oxygen species, which is likely to impair insulin signalling [14]. Hence, elevated production of Ang II might play an important role in the progress of insulin resistance, which precedes the development of type 2 *diabetes mellitus*. However, recent studies demonstrated the existence of two functionally opposing pathways of the RAS. The alternative pathway of the RAS involves the cleavage of Ang I or Ang II by ACE2 to Ang 1–7. Ang 1–7 acts via Mas receptor and has antagonistic effects to the classical RAS pathway [7].

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Local RASs found in several tissues are relatively physiologically and functionally independent of the systemic RAS, while they may actually contribute to the production of circulating angiotensin peptides [26, 27]. It has been demonstrated that skeletal muscle RAS is of physiological relevance since it possesses stretch-responsive activity [16]. Local production of several RAS family members has been revealed by the evidence of gene expression in muscle stem cells in vitro and was confirmed in skeletal muscle of rats [16], as well as in humans in vivo [20]. However, regulation of local muscle RAS, with respect to its complexity, in health and disease or patho/physiological conditions is so far unknown.

Regarding skeletal muscle physiology, endogenous Ang II may influence muscle performance and increase exercise-induced muscle hypertrophy [22]. On the other hand, chronic infusion of Ang II in high doses aggravates protein synthesis and enhances protein degradation, which might lead to muscle atrophy [4]. The strong influence of local Ang II production on skeletal muscle performance and composition is supported by studies investigating ACE gene polymorphism. The insertion (I) allele is associated with low ACE activity, and therefore low Ang II production, while the deletion (D) allele is linked to enhanced ACE activity. There is a linear trend between decreases in type I fibres and increases in type IIb fibres from ACE II to ID and to DD genotypes, which means from the lowest to the highest ACE activity [37]. The aim of our study was (i) to investigate the expression of the alternative RAS components and confirm the classical ones within skeletal muscle in vivo in Zucker rats and (ii) to examine the effect of obesity and (iii) aging on the expression of skeletal muscle RAS components and (iv) myosin heavy chain proportion.

Material and methods

Animals

Male Zucker fatty rats (*fa/fa*) ($n = 16$) and their lean controls (+/?) ($n = 13$) were purchased from Harlan (Udine, Italy). The animals were housed in a 12-h light/dark cycle with access to water and standard diet ad libitum. Intraperitoneal glucose tolerance test (IPGTT) was performed to assess glucose clearance. Overnight-fasted animals administered an i.p. injection of dextrose solution at a dose of 2 g/kg body weight. Glycaemia was measured in the tail vein blood immediately and in 30-min intervals for 2 h after glucose administration using a glucometer (Accu-Check Active, Roche diagnostics). After 2 days of recovery, overnight-fasted animals were killed at the age of 3 (young) resp. 8 (old) months by decapitation. Experimental procedures involving animals were approved by the Jagiellonian University Ethical Committee on Animal Experiments.

Transcardial perfusion surgery

In order to reveal the origin of renin protein expression in skeletal muscle, we performed tissue perfusion. The rats were deeply anaesthetized with an i.p. injection of pentobarbital (60 mg/kg, Spofa, United Pharmaceutical Works, Czech Republic, 500 mg) and then transcardially perfused through the aorta with 60 ml of 150 U/ml heparin in saline for 30 min.

Measurement of selected metabolic parameters

Circulating leptin and insulin levels were measured in plasma isolated from trunk blood samples obtained after decapitation using commercial radioimmunoassay kits (Millipore, Bedford, MA, USA) following the manufacturer's protocol. Lipid parameters were determined in the Laboratory Diagnostics Unit of The University Hospital in Krakow using commercially available kits (Roche Molecular Diagnostics, Pleasanton, CA, USA). Fasting glycaemia was analysed at SynLab (Bratislava, Slovakia) using multi-analyser COBAS Integra 800 (Roche Diagnostics Ltd. Rotkreuz, Switzerland). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as follows: fasting plasma glucose (mg/dl) × fasting plasma insulin (μU/ml)/2430 (Cacho et al., 2008).

RNA isolation and real-time PCR

Prior to sampling for RNA, dissected samples of quadriceps muscle were snap frozen and mashed under liquid nitrogen to powder. Samples were stored at − 80 °C until analyses. Total RNA was isolated from *musculus quadriceps* using RNeasy Universal Plus Mini Kit (Qiagen, Valencia, CA) and reverse transcription was performed using Maxima First-Strand cDNA Synthesis Kit (Thermo Fisher, Waltham, MA) according to the manufacturer's protocol. Real-time PCRs were carried out applying Maxima Sybr Green qPCR Master Mix (Thermo Fisher, Waltham, MA) and run on an ABI 7900HT thermal cycler (Applied Biosystems, Life Technologies, Carlsbad, CA) using rat-specific primer pairs shown in Table 1. Data were normalized to the expression of housekeeping gene ribosomal protein S29 (Rps29) which was altered neither by obesity nor age (Supplement 1B). Electrophoretic separation of PCR products was performed on a MGU-202T Horizontal Mini-Gel Kit equipment (C.B.S. Scientific, Del Mar, CA) in Tris-acetate-EDTA (TAE) buffer (Thermo Fisher Scientific, Rockford, IL). Samples were loaded with Orange DNA Loading Dye (Fermentas, Thermo Fisher Scientific, Rockford, IL) on a 1.5% agarose gel (Serva, Heidelberg, Germany) with GelRed nucleic acid stain (Biotium, Hayward, CA) added. Bands were visualized by BioDoc-It™ Imaging System (UVP, Upland, CA).

Table 1 Primer sequences for gene expression analysis using qPCR

Gene		Primer sequences used for qPCR
Ace	Fw	5′-ATG GTA CAG AAG AAG GGC TGG AA-3′
	Rv	5′-TTG TAG AAG TCC CAC GCA GA-3′
Ace2	Fw	5′-TCA GAG CTG GGA TGC AGA AA-3′
	Rv	5′-GGC TCA GTC AGC ATG GAG TTT-3′
Adam17	Fw	5′-GTG AGC AGT TTC TCG AAC GC-3′
	Rv	5′-AGC TTC TCA AGT CGC AGG TG-3′
Adam19	Fw	5′-AGG AAA GCA TCC ACT CAC GG-3′
	Rv	5′-GGA CGG GCT CGA TGA TGT AG-3′
Agt	Fw	5′-CAT GAG TTC TGG GTG GAC AA-3′
	Rv	5′-AAG TTG TTC TGG GCG TCA CT-3′
Apa (Enpep)	Fw	5′-GGC TCC CTT GTG GGT TTT TAC-3′
	Rv	5′-TCT TGT TGG GTT CAT CGA AAC A-3′
AT_1 (Agtr1a)	Fw	5′-TCT CAG CAT CGA TCG CTA CCT-3′
	Rv	5′-AGG CGA GAC TTC ATT GGG TG-3′
AT_2 (Agtr2)	Fw	5′-ACC TTT TGA ACA TGG TGC TTT G-3′
	Rv	5′-GTT TCT CTG GGT CTG TTT GCT C-3′
Furin	Fw	5′-GGG TTT CCC AGC AGT CTT CA-3′
	Rv	5′-GCC AGA TCC CCA GGT GTG-3′
Glut4	Fw	5′-TTT CCA GTA TGT TGC GGA TG-3′
	Rv	5′-TCA GTC ATT CTC ATC TGG CC-3′
Irap	Fw	5′-GGC ACA TCA GTG GTT TGG TAA TC-3′
	Rv	5′-TAC TCC ATG AAA GTG GCA AAG C-3′
Irs-1	Fw	5′-CCA AGG GCT TAG GTC AGA CAA A-3′
	Rv	5′-GCC TCA GAG TTG AGC TTC ACA A-3′
Mas1	Fw	5′-TGA CCA TTG AAC AGA TTG CCA-3′
	Rv	5′-TGT AGT TTG TGA CGG CTG GTG-3′
Myh1	Fw	5′-AAGACCGCAAGAACGTTCTC-3′
	Rv	5′-TCGTAAGTACAAAATGGAGTGAC-3′
Myh2	Fw	5′-TCCTCAGGCTTCAAGATTTG-3′
	Rv	5′-TTAAATAGAATCACATGGGGAC-3′
Myh4	Fw	5′-GAGGACCGCAAGAACGTG-3′
	Rv	5′-TGTGTGATTTCTTCTGTCACC-3′
Myh7	Fw	5′-AGAGGAAGACAGGAAGAACCTAC-3′
	Rv	5′-GGCTTCACAGGCATCCTTAG-3′
Nep (Mme)	Fw	5′-GCA GAA ATC AGA TCG TCT TCC CCG-3′
	Rv	5′-CTG AGT CCA CCA GTC AAC GAG GT-3′
Pgc1α	Fw	5′-CTT AAG TGT GGA ACT CTC TG-3′
	Rv	5′-CCT TGA AAG GGT TAT CTT GG-3′
Plzf (Zbtb16)	Fw	5′-GCGAAGAAGAAGAGGACCGTAAG-3′
	Rv	5′-CCGGAATGCTTCGAGATGAA-3′
Renin	Fw	5′-CCA CCT TCA TCC GCA AGT TC-3′
	Rv	5′-TGC GAT TGT TAT GCC GGT C-3′
Rer (Atp6ap2)	Fw	5′-TGG CCT ATA CCA GGA GAT CG-3′
	Rv	5′-AAT AGG TTG CCC ACA GCA AG-3′
Rps29	Fw	5′-GCTGAACATGTGCCGACACT-3′
	Rv	5′-GGTCGCTTAGTCCAACTTAATGAA-3′

Western blotting

Samples of *musculus quadriceps* were homogenized using a glass Teflon homogenizer in an ice-cold lysis buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1%SDS, 0.5 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 5 mg/ml leupeptin and 5 mg/ml aprotinin). Homogenates were placed on ice for 2 h with occasional mixing and centrifuged at 16,000×*g*/20 min/4 °C. The supernatant was used for western blot analysis. Protein concentration was determined by Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich St. Louis, MO) according to the manufacturer's instructions. Proteins were separated by SDS-PAGE technique on 10% polyacrylamide gels and electro-transferred in semi-dry conditions to a low-fluorescence PVDF membrane (Immobilon-FL, Millipore, Bedford, MA). The equal loading and transfer was confirmed by Ponceau staining (Serva, Heidelberg, Germany). Membranes were blocked in 5% milk in Tris-buffered saline for 1 h at room temperature and incubated overnight at 4 °C with primary antibody against (pro)renin receptor (Atp6ap2) (ab40790, Abcam, Cambridge, MA) diluted 1:2000, renin (ab180608, Abcam, Cambridge, MA) diluted 1:2000, IRAP (#6918, Cell Signalling Technology, Danvers, MA) diluted 1:1000, anti-myosin (Skeletal, Fast) (M4276, Sigma-Aldrich St. Louis, MO) diluted 1:1000, anti-myosin (Skeletal, Slow) (M8421, Sigma-Aldrich St. Louis, MO) diluted 1:4000 and/or beta-actin (#3700, Cell Signalling Technology, Danvers, MA) diluted 1:1000 in 5% milk or BSA in Tris-buffered saline. Beta-actin was used as endogenous loading control. After washing in TBS with Igepal the membranes were incubated with fluorescently labelled secondary anti-rabbit (#5151) or anti-mouse mouse (#5257) antibodies (Cell Signalling Technology) diluted 1:15000 for 1 h at room temperature. Infrared fluorescence was detected using the Odyssey infrared imaging system (LI-COR Biosciences), and Odyssey IR imaging system software version 2.0 was used for analysis.

Measurement of enzyme activity

Skeletal muscle was homogenized using a glass Teflon homogenizer in lysis buffer (250 mM saccharose, 10 mM Tris, pH 7.4). The homogenate was centrifuged at 1000×*g*/10 min/4 °C. The supernatant was collected and centrifuged at 16,000×*g*/15 min/4 °C to separate the membrane fraction. Protein concentration was measured by the bicinchoninic acid protein assay (Sigma-Aldrich, St. Louis, MO). Activity of aminopeptidase A (APA) was determined in the membrane fraction. The samples were mixed with substrate solution containing 10 mg/100 ml bovine serum albumin, 10 mg/100 ml dithiothreitol, 50 mM $CaCl_2$ in 50 mM Tris pH 7.4, and 100 mM H-Glu-β-naphthylamide (Bachem, Bubendorf, Switzerland), which served as a substrate for APA. The 96-

well was placed in Synergy™ H4 Hybrid Reader (Biotek, Winooski, Vermont, USA) fluorimeter and pre-heated to 37 °C. The enzyme kinetics was measured during 60 min in 5 min intervals as the amount of β-naphthylamide released from the substrate due to the enzyme activity of APA at wavelengths 340 nm (excitation) and 410 nm (emission). Enzyme activity is expressed in micromoles per litre of H-Glu-β-naphthylamide hydrolysed per minute per milligram of protein.

Statistical analysis

The results are presented as mean ± S.E.M. Analysis of normally distributed data was performed using Shapiro–Wilk test. Differences between experimental groups were analysed by two-way ANOVA with factors age and obesity. Whenever interaction between the main factors (age and obesity) reached significance, Bonferroni post-hoc test was applied. Non-normally distributed data were subjected to natural logarithm transformation prior to statistical analysis. Correlations between variables were analysed using Pearson correlation test. Overall level of statistical significance was reached at $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Results

Recessively homozygous (*fa/fa*) Zucker rats display obesity, hyperleptinaemia, hyperinsulinaemia, hypercholesterolaemia and hypertriglyceridaemia. We observed a significant rise in fasting glycaemia with age and obesity, however still within the physiological values. The progress of aging further deepened the metabolic disturbances in obese individuals. Aging, however, impaired the metabolic parameters of lean Zucker rats as well. In the case of body weight, plasma cholesterol and triglycerides levels, a significant interaction between age and obesity was detected, indicating that the obesity-related alterations were accelerated with aging (Table 2).

The expression of glucose transporter 4 (*Glut4*) was significantly downregulated by age ($p < 0.05$), while there was a significant interaction of age and obesity ($p < 0.01$) on insulin receptor substrate-1 (*Irs-1*) expression (Fig. 1a, b). The expression of *Irs-1* was significantly ($p < 0.001$) lower in the skeletal muscle of obese 3-month-old animals when compared to their age-matched lean controls. *Irs-1* expression subsequently decreased by age in lean animals as well ($p < 0.001$), so we can assume an early onset of decline in the expression of *Irs-1* by obesity. We examined the expression of insulin-regulated aminopeptidase (IRAP) on both mRNA and protein levels. The mRNA expression of *Irap* is significantly ($p < 0.05$) downregulated by aging (Fig. 1c), which corresponds with the age-related decline of *Glut4* expression. The protein content of IRAP was significantly decreased by obesity ($p < 0.05$) in the skeletal muscle of Zucker rats (Fig. 1d).

Components of the classical RAS pathway

The gene expression of angiotensinogen *(Agt)* was measured in *musculus quadriceps* of lean and obese Zucker rats at the age of 3 resp. 8 months (Fig. 2a). Two-way ANOVA revealed a significant interaction between the main factors: age and obesity. Bonferroni post hoc test showed significant downregulation of *Agt* gene expression in the skeletal muscle of lean 8-month-old Zucker rats compared to that observed in young controls ($p < 0.001$). Moreover, *Agt* gene expression was significantly reduced by obesity in the group of young animals ($p < 0.05$). However, this effect of obesity on *Agt* mRNA levels was not observed in 8-month-old rats.

There was a significant age-related decline of *Ace* gene expression in the skeletal muscle of Zucker rats while obesity had no significant effect (Fig. 2b) ($p < 0.05$).

In the case of angiotensin II type 1 receptor (AT_1), both aging and obesity significantly downregulated mRNA expression ($p < 0.05$) (Fig. 2c).

A significant interaction between the main factors age and obesity was detected regarding angiotensin type 2 receptor expression (AT_2) (Fig. 2d). Aging significantly decreased AT_2 mRNA levels in lean individuals ($p < 0.05$). However, a significant rise in AT_2 expression was observed in old obese Zucker rats in comparison with their lean controls ($p < 0.001$).

The expression of *Apa* was altered by neither age nor obesity. However, APA activity in the membrane fraction of skeletal muscle was significantly decreased in the obese Zucker rats in comparison with their lean controls (Fig. 3a) ($p < 0.001$). On the contrary, plasma APA activity was significantly elevated in obese individuals ($p < 0.001$). Interestingly, APA activity in the skeletal muscle negatively correlated with the APA activity in the plasma ($r = -0.54$, $p < 0.01$) (Fig. 3d). Furthermore, a significant negative correlation between plasma cholesterol and skeletal muscle APA activity was observed ($r = -0.43$, $p < 0.05$), whereas plasma cholesterol and plasma APA activity correlated positively ($r = 0.71$, $p < 0.001$).

Components of the alternative RAS pathway

Aging caused a significant decline in the expression of *Ace2* ($p < 0.05$). Furthermore, obesity showed a strong tendency to reduce *Ace2* mRNA levels ($p = 0.058$) in the skeletal muscle of Zucker rats (Fig. 4a). Similarly, the expression of the ACE2-shedding enzyme, ADAM metallopeptidase domain 17 (*Adam17*), was significantly downregulated by both obesity ($p < 0.05$) and aging ($p < 0.001$) (Fig. 4b).

Gene expression of Mas receptor (*Mas1*) measured in the skeletal muscle of Zucker rats was not significantly altered by age and/or obesity (Fig. 4c).

Table 2 Metabolic parameters of lean and obese Zucker rats at the age of 3 and 8 months

	3-Month-old		8-Month-old		Main effect		Interaction
	Lean (+/?) $n = 7$	Obese (*fa/fa*) $n = 8$	Lean (+/?) $n = 6$	Obese (*fa/fa*) $n = 8$	Age	Obesity	Age × Obesity
Body weight (g)	257.3 ± 4.9	388.3 ± 5.4 ***	457.3 ± 8.7♦♦♦	688.8 ± 17.9 ***,♦♦♦	$p < 0.001$	$p < 0.001$	$p < 0.001$
Leptin (ng/ml)	2.0 ± 0.1	35.9 ± 1.9	6.3 ± 0.7	94.4 ± 11.2	$p < 0.001$	$p < 0.001$	n.s.
Fasting glycaemia (mmol/l)	5.97 ± 0.15	6.34 ± 0.20	6.38 ± 0.17	6.94 ± 0.18	$p < 0.01$	$p < 0.05$	n.s.
Insulin (ng/ml)	0.5 ± 0.1	6.5 ± 0.7	1.4 ± 0.2	13.5 ± 1.8	$p < 0.001$	$p < 0.001$	n.s.
2 h Glycaemia (mmol/l)	4.18 ± 0.17	6.11 ± 0.36	6.26 ± 0.31	9.6 ± 0.89	$p < 0.001$	$p < 0.001$	n.s.
HOMA-IR	0.58 ± 0.14	8.71 ± 0.99	11.72 ± 1.47	120.54 ± 17.51	$p < 0.001$	$p < 0.001$	n.s.
Cholesterol (mmol/l)	2.23 ± 0.08	3.35 ± 0.13**	2.53 ± 0.06	6.86 ± 0.39 ***,♦♦♦	$p < 0.001$	$p < 0.001$	$p < 0.001$
Triglycerides (mmol/l)	0.74 ± 0.06	3.01 ± 0.22 ***	0.78 ± 0.04	4.46 ± 0.79 ***,♦♦	n.s.	$p < 0.001$	$p < 0.05$

HOMA-IR homeostasis model assessment of insulin resistance

Data are presented as mean ± S.E.M. and were analysed using two-way ANOVA with subsequent Bonferroni post hoc testing

The effect obesity: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ within the same age; effect of age ♦$p < 0.05$; ♦♦$p < 0.01$; ♦♦♦$p < 0.001$ within the same genotype

In the case of neutral endopeptidase (*Nep*) mRNA levels, a significant interaction between factors age and obesity was observed. As detected by the post hoc analysis, *Nep* gene expression was significantly downregulated in old lean rats

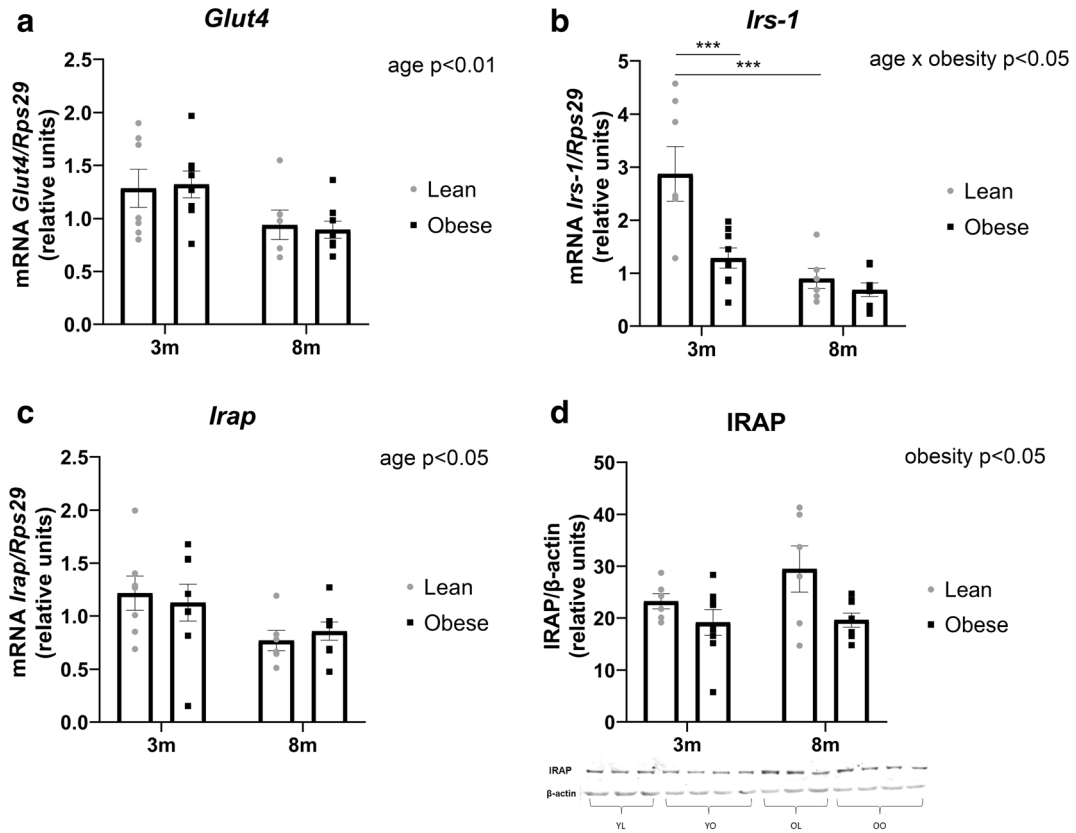

Fig. 1 Expression of glucose transporter 4 (*Glut4*), insulin receptor substrate-1 (*Irs-1*), and insulin-regulated aminopeptidase (*Irap*) at mRNA and/or protein levels. Expression of *Glut4* (**a**), *Irs-1* (Supplement 1A) (**b**), *Irap* at the level of mRNA (**c**) and protein (**d**) with representative western blot in *musculus quadriceps* of 3-month-old lean (*Fa/?*) ($n = 7$) and obese (*fa/fa*) ($n = 8$), resp. 8-month-old lean (*Fa/?*) ($n = 6$) and obese (*fa/fa*) ($n = 8$) Zucker rats. Levels of mRNA were determined by real-time PCR. Data were normalized to the gene expression of 40S ribosomal protein S29 (*Rps29*) whose expression was altered neither by obesity nor by age. The amount of IRAP protein was quantified by western blot method. β-Actin whose expression was altered by neither obesity nor age was used as endogenous loading control. Data presented as mean ± S.E.M. were analysed using two-way ANOVA, with subsequent Bonferroni post hoc test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

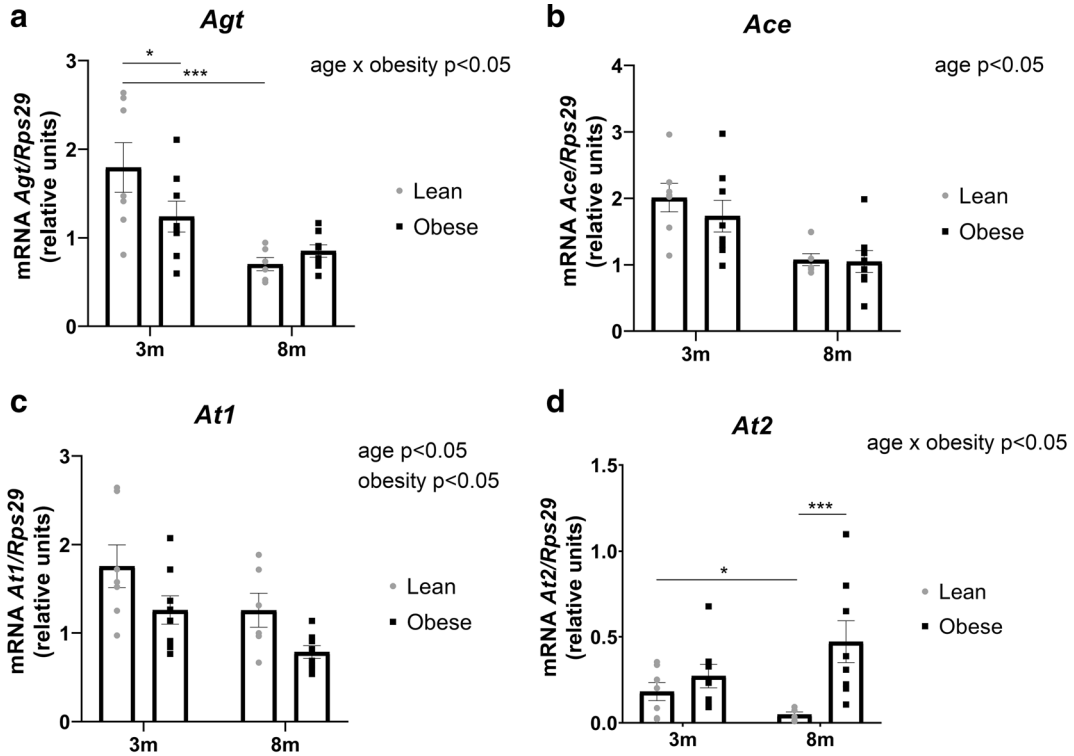

Fig. 2 Classical RAS pathway components in the skeletal muscle of Zucker rats. Gene expression of angiotensinogen (*Agt*) (Supplement 1A) (**a**), angiotensin-converting enzyme (*Ace*) (**b**), angiotensin II type 1 receptor (AT_1) (**c**) and angiotensin II type 2 receptor (AT_2) (**d**) in *musculus quadriceps* of 3-month-old lean (*Fa/?*) ($n = 7$) and obese (*fa/fa*) ($n = 8$), resp. 8-month-old lean (*Fa/?*) ($n = 6$) and obese (*fa/fa*) ($n = 8$) Zucker rats determined by real-time PCR. Data were normalized to the gene expression of 40S ribosomal protein S29 (*Rps29*) whose expression was altered neither by obesity nor by age. Data presented as mean ± S.E.M. were analysed using two-way ANOVA, with subsequent Bonferroni post hoc test; $*p < 0.05$; $***p < 0.001$

when compared to young controls ($p < 0.05$). In addition, mRNA levels of *Nep* were significantly increased in old obese rats compared to lean rats of the same age ($p < 0.05$). However, this effect of obesity was not observed in the group of young rats (Fig. 4d).

The renin/ReR pathway

The expression of renin was confirmed in skeletal muscle of Zucker rats at both mRNA and protein levels (Fig. 5). The mRNA levels of renin seem to be lower in skeletal muscle compared to the kidney (Fig. 5c). Paradoxically, an evidently higher signal for renin protein was detected in the skeletal muscle than that in the kidney of the same lean rat from the control group (Fig. 5d). Tissue perfusion was performed on Wistar rats in order to evaluate the effect of renin uptake. There was no evident difference in renin protein content in perfused samples when compared to non-perfused samples of *musculus quadriceps* (Fig. 5e). The effect of obesity on renin gene expression in the skeletal muscle depends on animal's age (Fig. 5a). Two-way ANOVA revealed a significant interaction between the factors age and obesity. Approximately 7-fold higher renin mRNA level was observed in the skeletal muscle of 8-month-old lean animals in comparison with their young controls ($p < 0.01$). Nevertheless, this increase of renin expression during aging was not present in the group of obese individuals. In consequence, mRNA levels of renin were significantly higher in lean 8-month-old rats compared to those found in the obese animals at the same age ($p < 0.001$) (Fig. 5a). However, there is an evident discrepancy in renin mRNA and protein levels. A significant interaction between factors age and obesity was found in the case of renin protein expression ($p < 0.001$) (Fig. 5b). Obesity lowered renin protein levels only in young individuals ($p < 0.001$). Furthermore, aging significantly decreased renin protein levels in lean individuals ($p < 0.001$), while it had an opposing effect in the obese ones ($p < 0.05$).

Statistical analyses revealed a significant main effect of obesity on mRNA levels of renin receptor (*ReR*) (Fig. 6a). Obesity resulted in a significant downregulation of the *ReR* ($p < 0.05$). Interestingly, an apparent discrepancy between renin receptor (ReR) mRNA and protein expression was observed (Fig. 6a, b). Two-way ANOVA revealed a significant interaction between the main factors. An age-dependent decline of ReR protein expression was observed in the skeletal muscle of lean animals ($p < 0.05$) but not of the obese ones. In consequence, ReR expression was significantly higher in obese 8-month-old rats in comparison with their age-

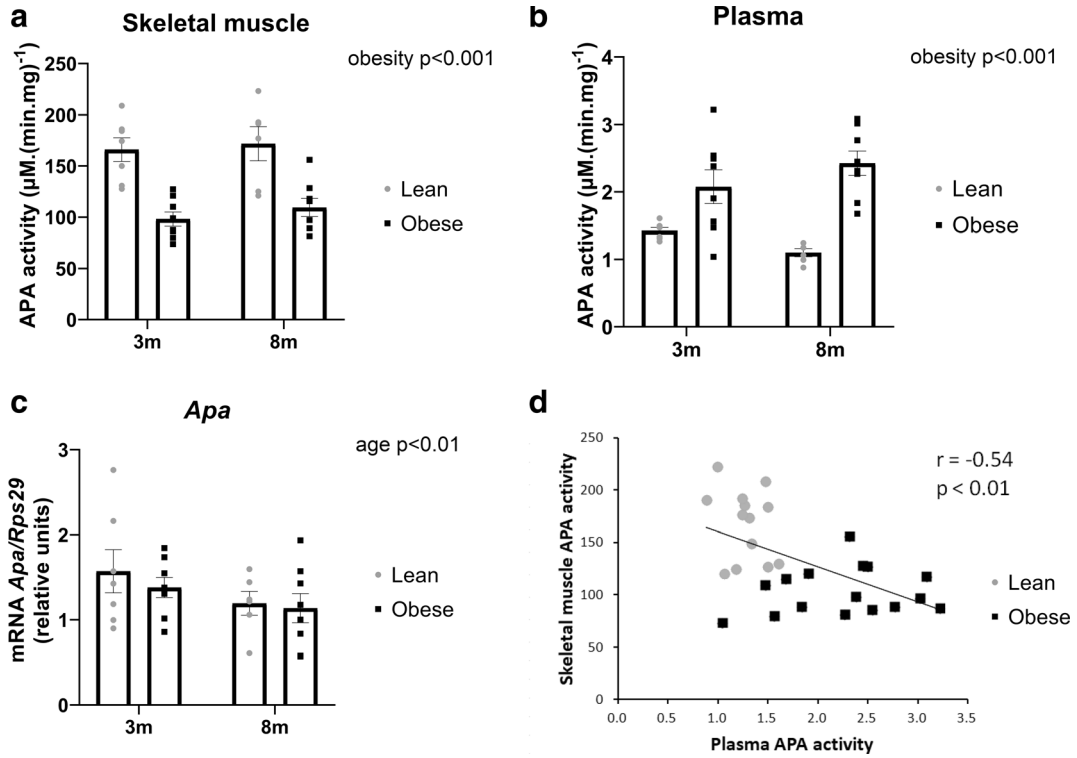

Fig. 3 Plasma and skeletal muscle APA activity. Enzyme activity of aminopeptidase A (APA) measured in the membrane fraction isolated from the skeletal muscle (**a**) and the blood plasma (**b**) of 3-month-old lean (*Fa/?*) ($n = 7$) and obese (*fa/fa*) ($n = 8$), resp. 8-month-old lean (*Fa/?*) ($n = 6$) and obese (*fa/fa*) ($n = 8$) Zucker rats. Gene expression of aminopeptidase A (*Apa*) in *musculus quadriceps* determined by real-time PCR (**c**). Data were normalized to the gene expression of 40S ribosomal protein S29 (*Rps29*) whose expression was altered neither by obesity nor by age. Data, presented as mean ± S.E.M. were analysed using two-way ANOVA. Correlation between plasma APA activity and skeletal muscle APA activity of 3-month-old lean and obese resp. 8-month-old lean and obese Zucker rats (**d**). Data were analysed using the Pearson correlation test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

matched lean controls ($p < 0.01$) (Fig. 6b). Furthermore, the mRNA levels of promyelocytic leukaemia zinc finger (PLZF) (*Zbtb16*), the repressor of ReR transcription, was examined (Fig. 6c). Two-way ANOVA found obesity-induced upregulation of PLZF gene expression ($p < 0.001$). We also studied the expression of proteolytic enzymes—*Adam19* and *furin*—involved in shedding of the ReR in the Golgi, producing the soluble form of ReR [6, 36]. The expression of *Adam19* was significantly downregulated by both aging and obesity ($p < 0.001$) (Fig. 6e), while in the case of *furin*, statistical analyses showed a significant interaction of age and obesity. In the lean animals *furin* mRNA levels were reduced by aging, while in the obese rats, there was an early onset of decreased *furin* expression (Fig. 6d).

Myosin heavy chain isoforms

The expression of *Myh7* characteristic for slow-twitch skeletal muscle fibre type 1 was significantly enhanced by obesity ($p < 0.01$) but not by aging (Fig. 7a). Aging lowered the expression of both *Myh4* ($p < 0.001$) and *Myh1* ($p < 0.001$) characteristic for fast-twitch fibre types 2A resp. 2B (Fig. 7c, d). The expression of *Myh2* (fast-twitch type 2X) was similarly downregulated by aging ($p < 0.001$), but significantly upregulated by obesity ($p < 0.001$) (Fig. 7b). A significant positive correlation was observed between *Myh2* ($r = 0.404$, $p < 0.05$), *Myh4* ($r = 0.653$, $p < 0.001$) and *Myh1* ($r = 0.526$, $p < 0.01$) expression in the skeletal muscle and the expression of ACE (Fig. 7f–i). Statistical analyses revealed a significant interaction between the main factors, age and obesity on mRNA levels of *Pgc1α* in skeletal muscle. Bonferroni post hoc test showed significant age-dependent decrease of *Pgc1α* expression in lean, but not obese animals. In consequence, the mRNA levels of *Pgc1α* were 2-fold higher in obese 8-month-old rats when compared to their lean controls (Fig. 7e). The expression of *Myh7* ($r = 0.503$, $p < 0.01$) positively correlated with the mRNA levels of *Pgc1α* (Fig. 7j). The protein content of fast myosin heavy chain (MyHC) isoform was significantly decreased by aging ($p < 0.05$), while there was a significant interaction of age and obesity ($p < 0.05$) on slow MyHC (Fig. 8a, b). The expression of slow MyHC was significantly elevated in the skeletal muscle of 8-month-old animals ($p < 0.05$), when compared to their lean controls.

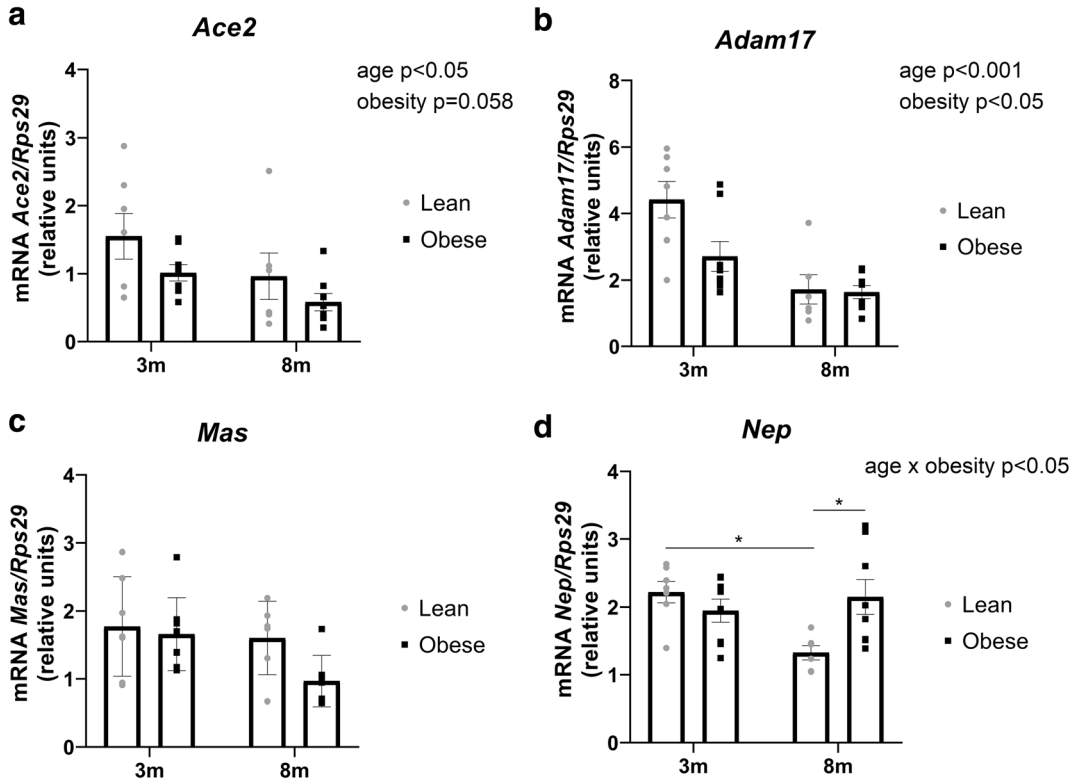

Fig. 4 Alternative RAS pathway components in the skeletal muscle of Zucker rats. Gene expression of angiotensin-converting enzyme 2 (*Ace2*) (**a**), *Adam17* (**b**) Mas receptor (*Mas1*) (**c**) and neutral endopeptidase (*Nep*) (Supplement 1A) (**d**) in *musculus quadriceps* of 3-month-old lean (*Fa/?*) ($n = 7$) and obese (*fa/fa*) ($n = 8$) resp. 8-month-old lean (*Fa/?*) ($n = 6$) and obese (*fa/fa*) ($n = 8$) Zucker rats determined by real-time PCR. Data were normalized to the gene expression of 40S ribosomal protein S29 (*Rps29*) whose expression was altered neither by obesity nor by age. Data presented as mean ± S.E.M. were analysed using two-way ANOVA, with subsequent Bonferroni post hoc test in the case of significant interaction between main factors, $*p < 0.05$; $***p < 0.001$

Discussion

Obese Zucker rats represent suitable model to study the prediabetic state characterized by peripheral insulin resistance, which is resulting in impaired insulin-stimulated glucose uptake in skeletal muscle [18]. Moreover, these rats display dyslipidemia, hyperinsulinemia [21] and a reduced microvessel density in the musculature [8]. Regarding the life span of the animals, lean male Zucker rats have a longer 50th percentile survivorship (816 days) and maximum life span (1067 days) than do obese Zucker rats (497 days 50th percentile survivorship, 803 (maximum life span)) [15]. In our study, we confirmed the age- and obesity-related metabolic alterations in the plasma of Zucker rats, accompanied by decreased gene expression of the insulin-dependent GLUT4 and an altered expression pattern of the downstream insulin signalling component, IRS-1, by age and obesity in the skeletal muscle. Since the design of the experiment required the use of overnight-fasted animals, the signal of GLUT4 protein was very weak and inconsistent in the skeletal muscle. For above reason, we analysed the expression of IRAP on both mRNA and protein levels, which is considered to be a surrogate marker for GLUT4 [11]. Similarly to GLUT4, the gene expression of IRAP was significantly downregulated by age, and we detected decreased protein content of IRAP by obesity.

A growing body of evidence indicates that dysregulation of the classical ACE/AngII/AT_1 pathway might contribute to the development of insulin resistance in the skeletal muscle by affecting insulin-mediated glucose uptake due to negative cross-interaction of Ang II with insulin receptor signalling pathway [14]. Furthermore, it has been proposed that the ACE2/Ang1–7/Mas axis acts antagonistically and that Ang 1–7 improves insulin sensitivity by increasing insulin-stimulated glucose uptake in the skeletal muscle in vivo [7]. Therefore, our purpose was to clarify the age- and obesity-related changes in the expression of skeletal muscle RAS components.

Gene expression of AGT, as well as that of ACE, was significantly downregulated with aging. Similarly, age-related decrease of AGT and ACE expression was reported in the adipose tissue of Wistar rats [19]. According to our results, we assume reduced ACE/Ang II/AT1 pathway activation in old obese individuals. Moreover, a significant age-related decrease and a strong lowering tendency ($p = 0.058$) of obesity on ACE2 gene expression were detected. Similarly, the expression of the ACE2-shedding enzyme, ADAM17,

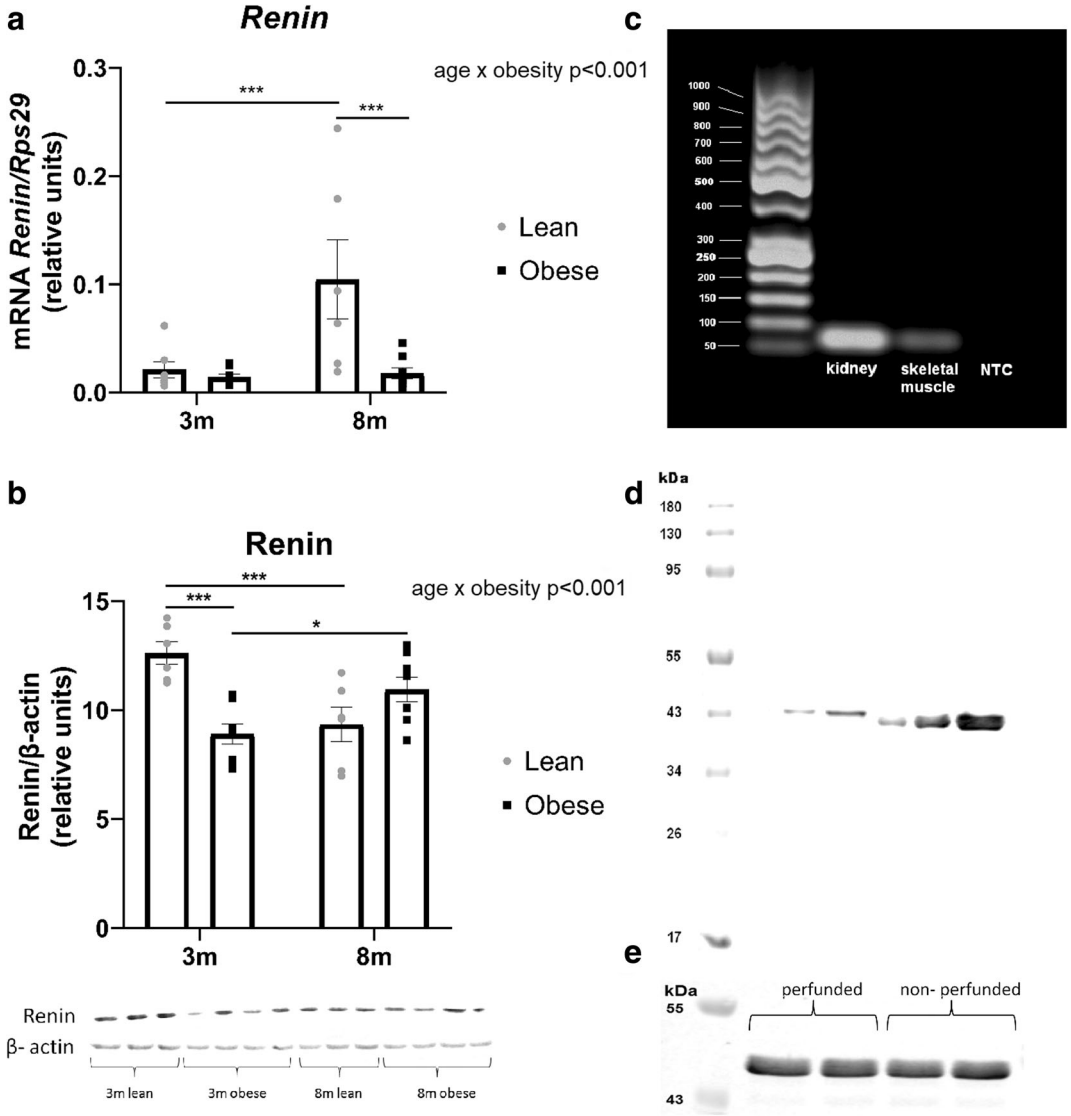

Fig. 5 Renin in the skeletal muscle of Zucker rats. Gene expression of renin (Supplement 1A) at the level of mRNA (**a**) and protein with representative western blots (**c**) in *musculus quadriceps* of 3-month-old lean (*Fa/?*) ($n = 7$) and obese (*fa/fa*) ($n = 8$), resp. 8-month-old lean (*Fa/?*) ($n = 6$) and obese (*fa/fa*) ($n = 8$) Zucker rats. Comparison of renin expression in skeletal muscle and kidney at both mRNA (**b**) and protein levels (**d**). Representative western blot comparing renin protein content in perfused and non-perfused *musculus quadriceps* of 3-month-old lean male Wistar rats (**e**). The same amount of cDNA (5 ng) obtained from skeletal muscle and kidney of the same lean individual from the 3-month-old group was loaded for PCR. Agarose gel electrophoresis was performed for PCR product separation and visualisation (**b**). Different amounts of total proteins (10 μg, 20 μg, 30 μg) isolated from skeletal muscle resp. kidney from the same lean control rat were loaded and determined by western blotting. mRNA levels were determined by real-time PCR. Data were normalized to the gene expression of 40S ribosomal protein S29 (*Rps29*) whose expression was not altered by obesity or age. The amount of renin protein was evaluated using western blot method. β-Actin whose expression was altered by neither obesity nor age was used as endogenous loading control. Data are presented as mean ± S.E.M. and were analysed using two-way ANOVA with subsequent Bonferroni post hoc test, $*p < 0.05$; $***p < 0.001$.

was significantly downregulated by both aging and obesity. Thus, the similar age- and obesity-related alterations in the expression of both classical and alternative RAS components suggest an overall attenuation of these two opposing pathways with the exception of AT2 and ReR receptors in obese animals.

The expression of AT_2 was demonstrated in the skeletal muscle in vitro [16] which was confirmed in our study in vivo. The expression pattern of AT_2 in the skeletal muscle of Zucker rats depends on the individual's phenotype. The characteristic age-related decline in the expression of AT_2 was disrupted by obesity and was significantly increased in the old obese individuals. Several studies have described up-regulated AT_2 expression under pathophysiological conditions in various tissues, e.g. diabetes, hyperglycaemia and metabolic syndrome [29]. It has been proposed that AT_2 receptors mediate diverse effects under normal and pathophysiological conditions [30]. Moreover, AT_2 receptors mediate

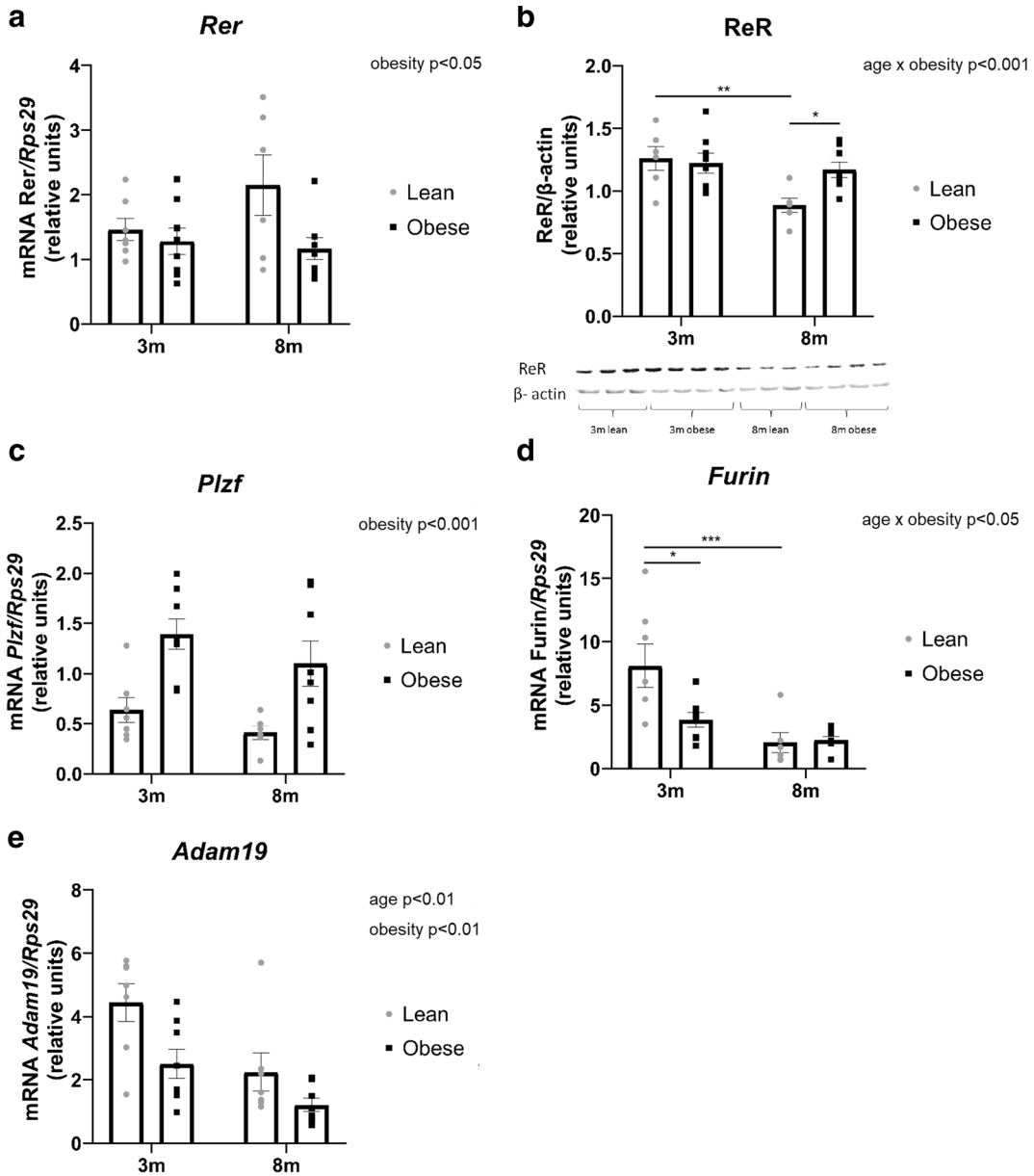

Fig. 6 Renin receptor in the skeletal muscle of Zucker rats. Gene expression of the renin receptor (*Rer*) at the level of mRNA (**a**) and protein (Supplement 1A) with representative western blots (**b**); mRNA expression of the transcription factor promyelocytic leukaemia zinc finger (*Plzf*, *Zbtb16*) (**c**), *furin* (Supplement 1A) (**d**), *Adam19* (**e**) in *musculus quadriceps* of 3-month-old lean (*Fa/?*) ($n = 7$) and obese (*fa/fa*) ($n = 8$) resp. 8-month-old lean (*Fa/?*) ($n = 6$) and obese (*fa/fa*) ($n = 8$) Zucker rats. Levels of mRNA were determined by real-time PCR. Data were normalized to the gene expression of 40S ribosomal protein S29 (*Rps29*) whose expression was altered neither by obesity nor by age. The amount of renin receptor protein was quantified by western blot method. β-Actin whose expression was altered by neither obesity nor age was used as endogenous loading control. Data are presented as mean ± S.E.M. Results were analysed using two-way ANOVA

anti-inflammatory and antioxidative functions in obese Zucker rats but proinflammatory and prooxidative functions in lean Zucker rats [30]. Regarding skeletal muscle physiology, AT_2 activation improves skeletal muscle perfusion, glucose metabolism and oxygenation [5].

It has been proven that Ang III is the predominant agonist for AT2 receptors [17]. APA (glutamyl aminopeptidase) is a membrane-bound enzyme, which hydrolyses Ang II to the heptapeptide Ang III [24]. The origin of the soluble form in the plasma has not been clarified yet, since there is no evidence for active secretion from none of the tissues. One possible mechanism for APA release is the autolysis of the membrane-bound enzyme [13]. Since fatty acid composition of skeletal muscle phospholipid bilayer is affected by plasma cholesterol levels, modifications in APA autolysis might occur due to elevated plasma cholesterol levels [12]. Interestingly, we detected a significant decrease of membrane-bound APA activity in the skeletal muscle of Zucker rats due to obesity,

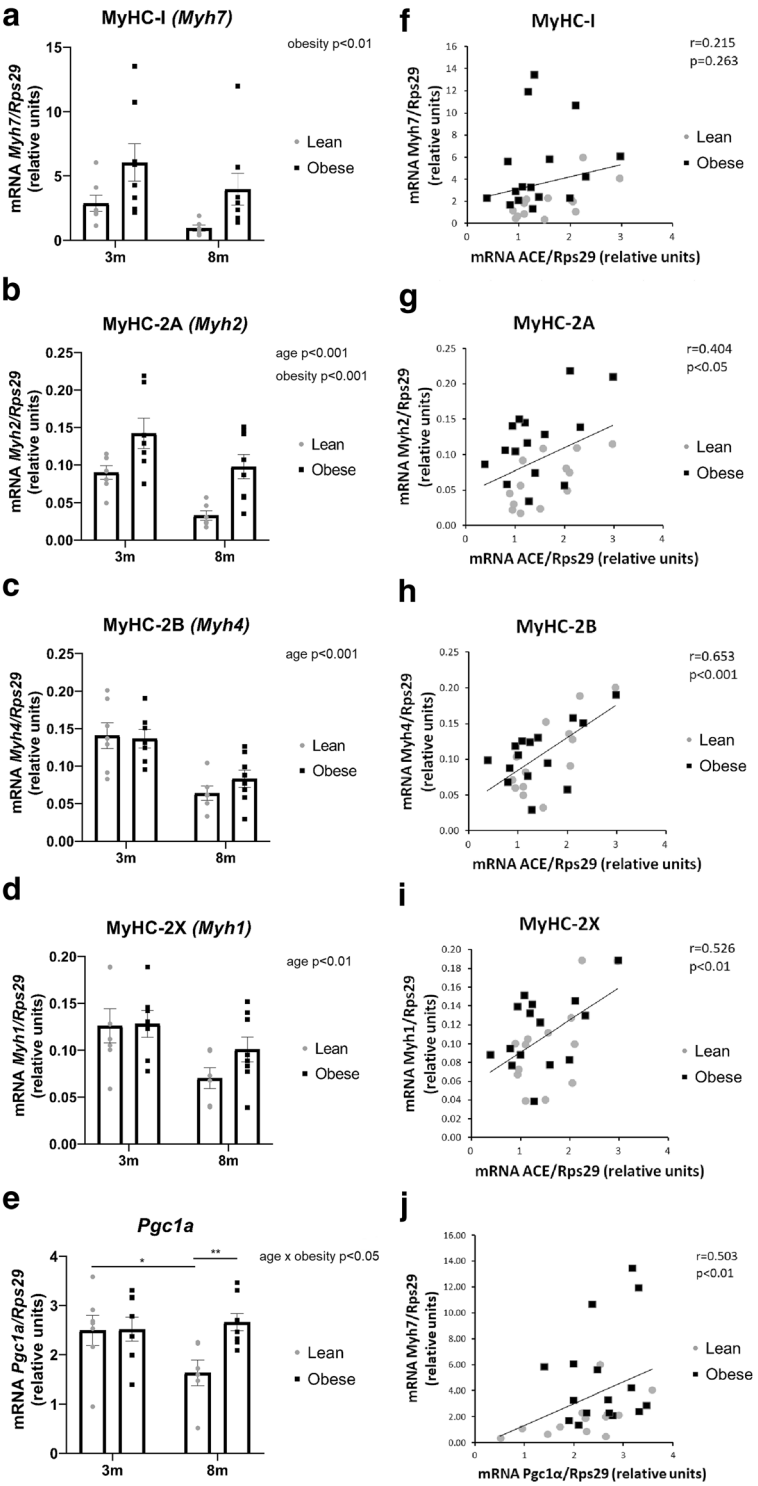

Fig. 7 Myosin heavy chain mRNAs, correlation with ACE expression. Gene expression of myosin heavy chain-slow (*Myh7*) (**a**), myosin heavy chain-2A (*Myh2*) (**b**) myosin heavy chain-2B (*Myh4*) (**c**) and myosin heavy chain-2X (*Myh1*) (**d**), coactivator proliferator-activated receptor γ coactivator 1 α (*Pgc1α*) (Supplement 1A) (E) in *musculus quadriceps* of 3-month-old lean (*Fa/?*) ($n = 7$) and obese (*fa/fa*) ($n = 8$) resp. 8-month-old lean (*Fa/?*) ($n = 6$) and obese (*fa/fa*) ($n = 8$) Zucker rats determined by real-time PCR. Data were normalized to the gene expression of 40S ribosomal protein S29 (*Rps29*) whose expression was altered neither by obesity nor by age. Data, presented as mean ± S.E.M. were analysed using two-way ANOVA. Correlation between skeletal muscle *Ace* and *Myh7* (**f**), *Myh2* (**g**), *Myh2* (**h**), *Myh1* (**i**); and between *Pgc1α* and *Myh7* (**j**) expression of 3-month-old lean and obese resp. 8-month-old lean and obese Zucker rats. Data were analysed using the Pearson correlation test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

which negatively correlated with the plasma APA activity. Simultaneously, the plasma cholesterol levels significantly correlated with the plasma APA activity. We may conclude that elevated plasma cholesterol might stimulate membrane-bound APA autolysis in the skeletal muscle, which in turn contributes to the rise of plasma APA activity in obese individuals. Another study suggests an elevated plasma APA activity in *ob/ob* mice when compared with their lean control, which resulted in elevated Ang III levels in the circulation together with enhanced natriuresis and AT2 receptor expression in the kidney [23]. The increased skeletal muscle AT2 expression old obese Zucker rats accompanied by elevated

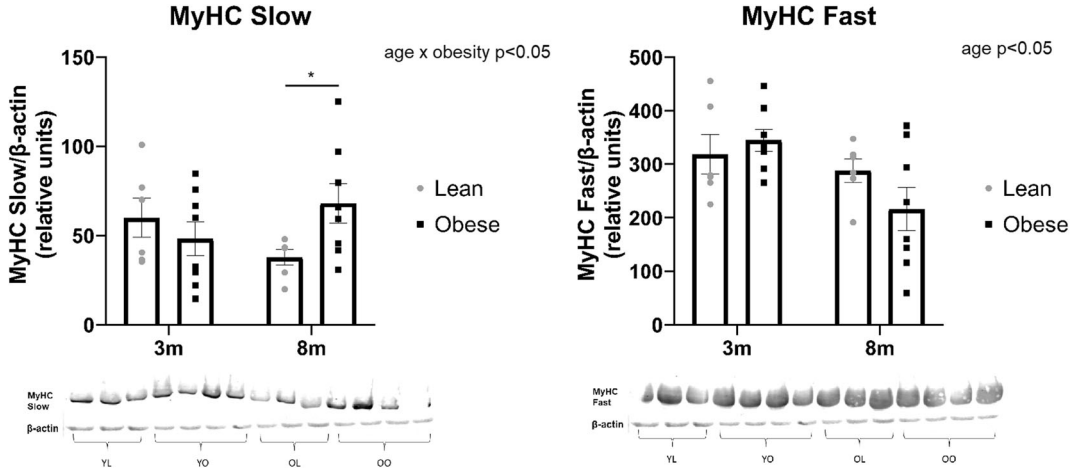

Fig. 8 Effect of age and obesity on myosin heavy chain protein proportion in the skeletal muscle. MyHC: slow 3-month-old lean 100% ± 18.26% and obese 80.26% ± 15.74%; 8-month-old lean 62.94% 8 ± 7.37% and obese 111.20% ± 17.52%; MyHC: fast 3-month-old lean 100% ± 11.71% and obese 108.19% ± 6.49%; 8-month-old lean 90.47% 8 ± 6.91% and obese 67.93% ± 13.67%. Protein expression of the slow (Supplement 1A) (**a**) and fast isoforms (**b**) of myosin heavy chain (MyHC) with representative western blots in *musculus quadriceps* of 3-month-old lean (*Fa/?*) ($n = 7$) and obese (*fa/fa*) ($n = 8$) resp. 8-month-old lean (*Fa/?*) ($n = 6$) and obese (*fa/fa*) ($n = 8$) Zucker rats. The amounts of MyHC proteins were quantified by western blot method. β-Actin whose expression was altered by neither obesity nor age was used as endogenous loading control. Data are presented as mean ± S.E.M. Results were analysed using two-way ANOVA

plasma APA activity assumes the activation of AngIII/AT2 axis as a part of a compensatory response to metabolic disturbances observed in these old obese individuals.

Neutral endopeptidase (NEP) is an integral membrane enzyme, which is involved in the degradation of various vasoactive peptides [28]. Within the RAS, NEP cleaves Ang I to Ang 1–7 and also, similarly to ACE, degrades bradykinin [10]. The lack of bradykinin has deleterious effects on insulin-mediated glucose uptake by the skeletal muscle [35]. Furthermore, NEP inhibition improves whole body insulin-mediated glucose disposal and insulin sensitivity in obese insulin-resistant Zucker rats by elevation in endogenous bradykinin [2]. We detected higher *Nep* mRNA levels in the skeletal muscle of old but not young obese Zucker rats in comparison with their age-matched controls. Elevated NEP expression might contribute to impaired glucose disposal revealed by the glucose tolerance test in the old obese Zucker rats.

Definitive proof for origin of renin expression within the skeletal muscle is still lacking. Some authors have not found any evidence of renin gene expression in primary myoblasts in vitro [16], while others suggest the existence of de novo renin production within the skeletal muscle of rats, although the detected product may actually be prorenin, as the antibody reacts also with the precursor molecule of renin [1]. Nevertheless, prorenin might contribute to local RAS activity by binding to renin receptor (ReR) and nonenzymatic activation [31]. We confirmed a local expression of renin at both mRNA and protein levels in the skeletal muscle. When compared to renin expression in the kidney on both levels, there was an evident discrepancy. The mRNA expression level of renin was lower, while the renin protein content was evidently higher in the skeletal muscle of the same control rat when compared to the kidney. To exclude excessive skeletal muscle renin uptake from the bloodstream, we determined renin protein in both perfused and non-perfused skeletal muscle samples of Wistar rats using western blot analysis with primary antibody against renin. However, there was no difference between the samples suggesting skeletal muscle renin origin. The detected higher renin mRNA level but lower signal for renin protein in the kidney versus skeletal muscle could be related to the fact that the majority of the kidney prorenin is the secretory product of the constitutive pathway, whereas only active renin is stored in renin granules and released by regulated pathway [33].

The gene and protein expressions of renin were affected by obesity. The age-induced increase of renin mRNA expression was eliminated by the progress of obesity. On the protein level, the expression of renin was influenced inversely by phenotype during aging. The renin protein expression significantly decreases in lean and rises in obese individuals with aging. In light of our findings and the fact that the ReR is expressed in the skeletal muscle, we emphasize the importance of paracrine/autocrine regulation of prorenin/renin/ReR axis in skeletal muscle.

In our study, we detected an evident discrepancy of ReR expression on mRNA and protein levels. ReR is able to directly interact with the transcription factor PLZF and inhibit its own expression [31]. We revealed the existence of the ReR/PLZF ultrashort negative feedback loop in the skeletal muscle of 8-month-old rats by determination of elevated ReR gene expression and decreased ReR protein and PLZF gene expression. Furthermore, we measured the expression of ReR-cleaving enzymes, furin and ADAM19. Furin is an

intracellular serine protease, which cleaves ReR after the Golgi compartment and a small proportion of full-length ReR is addressed to the plasma membrane, while the soluble form is secreted from the cells [6]. On the other side, furin may not be pivotal in the shedding of ReR. ADAM19 is another enzyme responsible for the cleavage of ReR in the Golgi and release of its soluble form in to the extracellular space [36]. The expression of furin is unchanged between lean and obese 8-month-old animals. However, the expression of ADAM19 is significantly lower in aged obese animals in comparison with the lean ones, which might contribute to an impaired ReR shedding and elevated ReR protein content in the skeletal muscle of these animals. Moreover, ReR in the skeletal muscle is involved in the development of insulin resistance by enhancing oxidative stress [9]. Significantly higher ReR protein expression in the old obese Zucker rats suggests the renin/ReR pathway over-activation in late-stage obesity.

To our knowledge, there is a lack of evidence regarding the relationship of ACE expression and fibre type composition in the skeletal muscle during the development of obesity and aging. It is generally accepted that skeletal muscle fibre types are related to four MyHCs encoded by specific genes, and real-time PCR method can be used as a routine method for analysis of muscle composition changes [38]. In this study, we investigated the relationship between skeletal muscle MyHC composition [38] and ACE gene expression during aging and the development of obesity. The expression of MyHC characteristic for fast-twitch muscle fibres type 2A (encoded by *Myh1*), type 2B (encoded by *Myh4*) and type 2X (encoded by *Myh2*), but not for the slow-twitch muscle fibre type 1 (encoded by *Myh7*) positively correlated with the expression of ACE. Our results suggest that the decreased expression of ACE is associated with decreased proportion of fast-twitch fibres in the aging skeletal muscle. This is in accordance with studies investigating ACE I/D gene polymorphism, where the muscle fibre type 1/type 2 ratio increases upon decreasing ACE activity [37]. Furthermore, aging is associated with a shift from fast to slow fibre type, affecting mostly type 2X fibres [25]. It is important to note that in obesity, the expression of oxidative muscle fibre types 1 and 2A is significantly enhanced, while the expression of both glycolytic muscle fibre types 2B and 2X is not affected by obesity. These changes on the mRNA level correspond to the altered protein expression of slow and fast MyHCs either by age and/or obesity. This suggests that by aging, there is a natural shift in the composition of skeletal muscle towards the oxidative muscle fibres, which is accelerated by obesity. Several lines of evidence show the increased abundance of slower oxidative type 1 in the skeletal muscles of genetically obese mice which might be explained by the greater functional demand on postural muscles with the increasing weight of obese animals [32]. Furthermore, we measured the expression of the transcriptional coactivator proliferator-activated receptor γ coactivator 1 α (PGC1α), which controls the expression of genes involved in oxidative phosphorylation, mitochondrial biogenesis and other features of oxidative muscle fibres and exerts anti-inflammatory effects [34]. We found that the expression of PGC1α is elevated in the skeletal muscle of aged obese rats and significantly correlates with the expression of *Myh7*, slow-twitch oxidative muscle fibre type 1. Our data show an age- and obesity-induced shift in MyHC proportions from fast- to slow-twitch fibres, which might be a part of compensatory mechanism counteracting the deleterious metabolic effects occurring in the skeletal muscle during aging and obesity.

In summary, our study confirmed the expression of both classical and alternative RAS components in the skeletal muscle of Zucker rats in vivo and have indicated their an overall age-related decrease in the skeletal muscle of Zucker rats with the exception of AT2 and ReR in obese animals. Obesity combined with aging had a more adverse effect on the expression pattern of several RAS components than obesity alone in young individuals. Observed increase in APA release from the skeletal muscle in obesity might contribute to increased plasma APA activity. Furthermore, we confirmed the existence of the ReR/PLZF ultrashort loop negative feedback mechanism, which was activated by obesity. It seems that age and obesity set up a new lower level equilibrium between classical and alternative RAS in skeletal muscle accompanied by increased renin/ReR/PLZF pathway activation. Our results show that by aging, there is a shift in the composition of skeletal muscle from fast-twitch glycolytic muscle fibres towards the oxidative muscle fibres, which is accelerated by obesity.

Although being a descriptive study, our data brings insight into complex regulation of the whole renin angiotensin system within the skeletal muscle thereby fulfilling a gap in this field. Evidence for the local RAS dysregulation in obesity and aging provided by us could stimulate design of subsequent intervention or functional studies aimed to decipher effect of individual angiotensins on muscle metabolism and composition.

Author contributions V.L. prepared major parts of the manuscript. V.L. and L.B. carried out western blots analyses, measurements of enzyme activity, conducted real-time PCR analyses and data interpretation. M. S. and L.B. conducted glucose tolerance tests and biochemical analyses. L.H. carried out transcardial perfusion surgery. L.B., K.K. and S.Z. supervised and edited the manuscript. R. O. and S.Z. planned and organized the study and contributed to the revisions and the final drafts of the manuscript. All authors have read and approved the final manuscript.

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Compliance with ethical standards

Experimental procedures involving animals were approved by the Jagiellonian University Ethical Committee on Animal Experiments.

Conflict of interest The authors declare that there is no conflict of interest.

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