Senescent murine femoral arteries undergo vascular remodelling associated with accelerated stress-induced contractility and reactivity to nitric oxide

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Abstract
This work explored the mechanism of augmented stress-induced vascular reactivity of senescent murine femoral arteries (FAs). Mechanical and pharmacological reactivity of young (12–25 weeks, y-FA) and senescent (>104 weeks, s-FAs) femoral arteries was measured by wire myography. Expression and protein phosphorylation of selected regulatory proteins were studied by western blotting. Expression ratio of the Exon24 in/out splice isoforms of the regulatory subunit of myosin phosphatase, MYPT1 (MYPT1-Exon24 in/out), was determined by polymerase chain reaction (PCR). While the resting length–tension relationship showed no alteration, the stretch-induced tone increased to 8.3 ± 0.9 mN in s-FA versus only 4.6 ± 0.3 mN in y-FAs. Under basal conditions, phosphorylation of the regulatory light chain of myosin at S19 was 19.2 ± 5.8% in y-FA versus 49.2 ± 12.6% in s-FA. Inhibition of endogenous NO release raised tone additionally to 10.4 ± 1.2 mN in s-FA, whereas this treatment had a negligible effect in y-FAs (4.8 ± 0.3 mN). In s-FAs, reactivity to NO donor was augmented (pD2 = −4.5 ± 0.3 in y-FA vs. -5.2 ± 0.1 in senescent). Accordingly, in s-FAs, MYPT1-Exon24-mRNA, which is responsible for expression of the more sensitive to protein-kinase G, leucine-zipper-positive MYPT1 isoform, was increased. The present work provides evidence that senescent murine s-FA undergoes vascular remodelling associated with increases in stretch-activated contractility and sensitivity to NO/cGMP/PKG system.

Keywords
ageing, cardiovascular pharmacology, cardiovascular pharmacology, hypertension, hypertension, reproductive and developmental toxicology

Lubomir T. Lubomirov and Monique Heidrun Jänsch contributed equally to this study.

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Arterial stiffness increases as part of the ageing process. Besides senescence, it is well established that the presence of cardiovascular risk factors augments arterial stiffness. Epidemiological studies suggest that hypertension accelerates the process of arterial stiffening in ageing. The mechanisms associated with increased arterial stiffness involve a number of components within the vascular wall in addition to ageing, such as mechanical stimuli, calcification, inflammatory cells and cytokines, changes in the properties of the ECM, sex, alterations in smooth muscle function, endothelial effects as NO release and other disease-related processes like stroke-induced dysfunction by sympathetic overactivity. A large body of evidence indicates that a high artery stiffness leads to left ventricular (LV) remodelling and has adverse consequences on LV function by increasing afterload. A high arterial stiffness is independently associated with diastolic dysfunction in clinical and community-based cohorts and strongly predicts heart failure (HF). In clinical trials, stiffness of large arteries emerged as trigger for HF with preserved ejection fraction (HFpEF).  

The femoral artery (FA) is a large conduit vessel from the lower limb circulation, formed in humans after completion of the iliofemoral system. Like in other mammalian conduit vessels, it responds to various contractile stimuli, i.e., α1-adrenoreceptor agonists, 5-hydroxytryptamin, endothelin-1, eicosanoids, NO inhibition, etc. It is common knowledge that basal and agonist-induced contractility of the FA could be altered by pathological conditions such as hypoxia, gram-negative bacterial endotoxins, diabetes or microgravity. Augmented vascular tone often coincides with advanced age, as at present, it is well appreciated that ageing increases muscle sympathetic nerve activity. It has also recently been shown that on the level of smooth muscle cells, ageing induces a diminution of the Ca2+ -dependent chloride conductance and a rise in agonist-induced contractility. Another study on vascular tissue from diabetic patients reported a development of a hypercontractile phenotype of the vessels from femoral region used as a vascular conduit in coronary artery bypass grafting, as authors reported that the underlying mechanism relates to a diminution of the activity of the enzyme myosin light chain phosphatase (MLCP). A causal link between ageing, impaired vasodilator responses and endothelial dysfunction in large conducting vessels has been reported by others. Altogether, these data support the view that ageing affects all components of vascular regulation, i.e., neuronal, endothelial and smooth muscle cells, leading to development of an abnormal vascular phenotype, often associated with hypercontractility.

Despite the functional importance of the FA for the blood supply in lower limb and, in general, for the whole circulatory system, comprehensive data regarding the basic mechanisms for regulation of contractility in this vascular bed are scarce. The present study aimed to explore the mechanisms of regulation of vascular contractility of FAs in senescence, as increased vascular tone might pave the way for HFpEF, stroke, kidney failure and other systemic dysfunctions. Herein, we report that senescence augments the contractile response of FA after mechanical stretch along with an increased basal phosphorylation level of 20-kDa regulatory light chain of myosin at S19 (MLC20-S19) and changes of the expression pattern of Exon 24 of the targeting subunit of myosin phosphatase, MYPT1.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All experiments were performed in accordance with the European Community guidelines (Directive 2010/63/EU) and approved by the State Office for Nature, Environment and Customer Protection North Rhine Westphalia: AZ 84-02.05.50.15.029). C57BL/6N young adult (y-FA, 12–25 weeks, n = 24) or adult senescent mice (s-FA, ≥104 weeks, n = 25) were bred and kept in the Center for Molecular Medicine Cologne and randomly allocated to the different sets of experiments. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.

2.2 | Tissue preparation and contractile protocols

These experimental series were performed with minor modification as in Welter et al. Detailed description of tissue preparation, all contractile protocols and solutions can be found in the Supporting Information.

2.2.1 | Protein extraction and immunoblotting

Proteins for immunoblotting assays were extracted as described in Lubomirov et al. In brief, FAs with intact endothelium or mechanically de-endothelialized (in these vessels, endothelium was removed by gently rubbing of internal vascular lumen by wire) were mounted on 25-μm tungsten wires in HEPES-buffered...
physiological salt solution (HPSS) and then equilibrated in carbogen aerated PSS for 10–15 min. Afterwards, the temperature was increased to 37°C, and the arteries were incubated either with 3 μmol/L Rho-kinase inhibitor, Y27632 or 100 μmol/L pan-inhibitor of nitric oxide synthase (NOS), Nω-nitro-ω-arginine methyl ester (L-NAME) or vehicle (H2O; time matched controls) for 10 min. Thereafter, preparations were shock frozen in 15% trichloroacetic acid in acetone, precooled with dry ice and fixed at −80°C for 3 h or overnight. Specimens were rinsed several times with acetone, precooled with dry ice and fixed at −80°C for 3 h or overnight. Specimens were homogenized in extracting buffer (see solution section in the Supporting Information) using glass homogenizers (Kimble Chase LLC, Tissue Grinder Micro PKG/6, art. No. 885470-0000) and the proteins were extracted for 1 h on ice. Then, homogenates were centrifuged at 20,000×g for 10 min, and the supernatants were subjected to SDS-PAGE (4%–20% gradient). Proteins were then transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membranes in Towbin-transfer-buffer, precooled with ice, at 22 V overnight. The membranes were blocked with 2% milk in Tris-buffered saline solution containing 0.05% Tween 20 (TBST) for 1 h. Thereafter, the membranes were incubated for 4 h at room temperature or overnight at 4°C with respective primary antibodies in 2% milk/TBST, except those incubated with anti-pMLC20-S19, where incubation was performed in 3% bovine serum albumin/TBST. Equal loading was verified by incubating the membranes with anti-MYPT1-total, anti-α- or -β-actin, anti-GAPDH, or SM-22 antibodies. As secondary antibodies, HRP-conjugated anti-rabbit, anti-mouse or anti-goat antibodies were used. Immunoreactive signals were visualized using enhanced chemiluminescence (West Pico or West Dura, from Pierce) and detected with the Chemi Premium Imager (VWR, Darmstadt, Germany). Quantification and analysis were performed with the Gel Documentation software (VWR).

2.3 | Antibodies

A detailed list of all antibodies used in this study can be found in the Supporting Information.

2.4 | Determination of expression ratio of MYPT1 Exon24-in/out mRNA by quantitative PCR (qPCR)

Total RNA of young and senescent FAs was extracted using the High Pure RNA Isolation Kit (Roche, # 11 828 665 001; Rotkreuz, Switzerland) following the manufacturer’s instructions. Initial grinding of the tissue was performed with glass homogenizers, using the supplied lysis buffer. RNA concentrations were quantified by their optical densities using a NanoDrop device (Thermo Fisher Scientific). Up to 1-μg RNA was used for first strand cDNA synthesis (iScript cDNA Synthesis Kit, Bio-Rad). For detection of the two MYPT1 splice variants (+/− Exon), the cDNA was amplified by polymerase chain reaction (PCR) with primers flanking Exon 24. PCR reactions were performed in final volumes of 20 μL, containing: 50-ng cDNA as template, 1× PCR buffer, 2.5 mmol/L MgCl2, 200 μmol/L dNTPs, each (Thermo Fisher Scientific), 2.5 U Taq DNA Polymerase (QIAGEN, Hilden, Germany), and 0.2 μmol/L forward 5′-ATT CCT TGC TGG GTC GCT CTG C-3′ respectively reverse 5′-ATC AAG GCTCATTTTCCATCC-3′ primer.5,18 The reactions were carried out on a thermocycler peqSTAR device (Peqlab Life Science/VWR) following a typical PCR cycling programme with 30 cycles and 57.5°C annealing temperature. PCR products were electrophoresed on 3% agarose gels (100 V for 1 h). For analysis, a 50-bp peqGOLD ladder (VWR Chemicals) was loaded. Amplification products were detected by UV on the CHEMI Premium imager (VWR Collection), and the MYPT1-Exon 24-in/out expression ratio was determined with the gel documentation software (VWR).

2.5 | Statistics

Results are given as mean ± SEM. The values of Fmax are given as absolute force in (mN). pD2 values of all relaxing compounds were calculated by Graph Pad software using the following equation: 

\[ Y = \text{Bottom} + \left(\text{top-bottom}/(1 + 10^{(X-\log IC50)})\right) \]

If not indicated otherwise, n represents the number of animals. The significance level was set at p < 0.05 and was tested with Student t test when two pairs were compared or with analysis of variance (ANOVA) for multiple comparisons followed by Tukey multiple comparisons post-test.

3 | RESULTS

3.1 | Senescent FAs develop stretch-induced tone

Firstly, we explored the development of stretch-induced tone (SIT) of y- and s-FA in order to test whether senescence affects basal vascular contractility. When the mounted vessels were stretched stepwise to IC90 (see Section 2), there was no significant difference in passive length–tension relationships between y- and s-FAs.
(Figure 1A,B). However, 2–5 min after stretch, s-FAs developed 8.3 ± 0.9 mN SIT as compared to only 4.6 ± 0.3 mN measured in y-FAs (Figure 1A–C; Table 1). We also analysed length–tension relationships and SIT under Ca$^{2+}$-free conditions (2 mmol/L EGTA). In both age groups, 10-min pretreatment with 2 mmol/L EGTA induced a small rightward shift of length–tension relationships. While in y-FAs, IC90-values at PSS and Ca$^{2+}$ free were similar, in s-FAs, EGTA reduced SIT close to the levels measured in y-FAs ($n = 4$; Figure 1B,C).

### 3.2 Reactivity to U46619, acetylcholine and Y27632 in y- and s-FAs

We then tested whether senescence alters reactivity of y- and s-FAs to the thromboxaneA$_2$-receptor agonist, U46619. After 20-min equilibration and treatment with vehicle (H$_2$O) or pan-NOS-inhibitor L-NAME, preparations were stimulated with cumulative additions of U46619. In s-FAs an inhibition of spontaneous NO release by 100 μmol/L L-NAME further increased tone to 10.4 ± 1.7 mN, whereas in y-FAs, tone amounted to only 4.8 ± 0.3 mN (Table 1). In s-FAs, in vessels pretreated with L-NAME, U46619 dose responsiveness shifted leftward (Figure 1D–F; Table 1). Furthermore, in s-FAs, the EC$_{50}$ value was significantly lower, and the maximal force was higher as compared to those values measured in y-FAs (Table 1).

We also tested whether senescence affects endothelium-dependent relaxation of FAs. After maximal cumulative stimulation with U46619, vessels were relaxed in PSS and further stimulated submaximally by 0.3 μmol/L U46619. After stabilization of U46619-induced contraction, acetylcholine was added cumulatively in concentrations 0.001–10 μmol/L. In y-FAs, acetylcholine induced a biphasic relaxation, characterized by a fast and a slow phase (Figure 2A,B). In

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**FIGURE 1** Passive length–tension relationships, stress- and U46619-induced tone in young mature (y-FA) and senescent (s-FAs) femoral arteries. (A) Original force tracings representing the normalization procedure at young (black) and senescent (red) femoral arteries. (B, C) Statistic evaluation of passive length–tension relationships (B) and initial stress-induced tone (C) during the equilibrium time of 15–20 min. (D–F) Original force tracings and statistic summary of the cumulative effect of U46619 in femoral arteries from both age groups under control conditions and after inhibition of NO by 100 μmol/L LNAME ($n = 8$).
senescent FAs, the fast relaxation was absent and the slow relaxation component was attenuated (Figure 2A,B; Table 2). In a separate experimental approach, we tested, whether NO release from vascular endothelium contributes to acetylcholine-induced relaxation. For this, vessels were treated with L-NAME, prior to submaximal stimulation. While in s-FAs, L-NAME reduced acetylcholine relaxation to 9.1 ± 0.7% (Figure 2C,D; Table 2). All contractile parameters of y-FAs, treatment with Y27632 had no effect on MLC20 phosphorylation (Figure 3A,C). Treatment with 3 μmol/L Y27632 reduced this parameter to levels measured in young vessels, while in y-FAs, treatment with Y27632 had no effect on MLC20-S19 phosphorylation (Figure 3A,C).

3.4 | Effect of senescence on markers of the activity of MLCP

In order to examine whether the observed increase in MLC20 phosphorylation relates to a reduction of MLCP activity, we first tested whether senescence might directly inhibit MLCP by phosphorylation of C-kinase potentiated protein phosphatase-1 inhibitor (CPI-17). In the vascular system, this protein has been shown to directly inhibit MLCP, when phosphorylated at T38.20 Under control conditions, the immunoreactive signal of CPI-17-pT38 was negligible and Y27632 treatment had no effect on this parameter (Figure 3A,B). Significant increase in the CPI-17-pT38 immunoreactive signal was detected only in samples treated with 0.1 μmol/L phosphatase inhibitor calyculin (Figure 3A,B), supporting the notion that phosphorylation of this regulatory protein relates to changes in the contractile state induced predominantly by agonist stimulation.

We then explored whether senescence would increase phosphorylation of the MLCP targeting subunit (MYPPT1) at amino acids T696 and T853. It has been shown

### TABLE 1
Contractile parameters of young and senescent FAs stimulated by cumulative concentrations of TromboxaneA2-receptor agonist, U46619

| Treatment             | Stretch-induced tone 20-min PSS | Stretch-induced tone (+20-min treatment with 1% H2O (control) or 100 μmol/L L-NAME) | $F_{\text{max}}$ (mN) | $-\log[EC_{50}]$ | n |
|-----------------------|-------------------------------|------------------------------------------------------------------------------------------------|------------------------|-----------------|---|
| U46619_Young (control) | 4.5 ± 0.2                     | 4.5 ± 0.3                                                                                     | 20.4 ± 1.4             | 7.2 ± 0.05      | 8 |
| U46619_Young (L-NAME)  | 4.6 ± 0.3                     | 4.8 ± 0.3                                                                                     | 20.2 ± 1.2             | 7.5 ± 0.09      | 8 |
| U46619_Senescent (control) | 7.4 ± 0.8*                  | 7.2 ± 0.7                                                                                     | 25.7 ± 1.0             | 7.3 ± 0.08      | 8 |
| U46619_Senescent (L-NAME) | 8.3 ± 0.9**                  | 10.4 ± 1.2                                                                                    | 24.8 ± 1.2             | 7.8 ± 0.1**     | 8 |

Note: n.s. = p = 0.139; $-\log[EC_{50}]$ U46619_Young (control) versus U46619_Young (L-NAME); two-way ANOVA. p = 0.0424; $-\log[EC_{50}]$ U46619_Young (L-NAME) versus U46619_Senescent (L-NAME); two-way ANOVA. n.s.: p = 0.0683; $F_{\text{max}}$ Young (L-NAME) versus Senescent (L-NAME); two-way ANOVA.

3.3 | Senescence increased resting MLC20-S19 phosphorylation of FAs

We then tested the effect of senescence on pathways responsible for contractile regulation. In these experimental series, isolated rings from y- and s-FAs were subjected to western blot analysis. Since ageing might also increase the sensitivity of peripheral arteries towards ROK inhibition,19 we compared phosphorylation level of MLC20 under control conditions (PSS, no stimulation) and after ROK inhibition by treatment with 3 μmol/L Y27632. We found that senescence increased basal phosphorylation of MLC20-S19, the site that primarily regulates contraction (Figure 3A,C). Treatment with 3 μmol/L Y27632 reduced this parameter to levels measured in young vessels, while in y-FAs, treatment with Y27632 had no effect on MLC20-S19 phosphorylation (Figure 3A,C).
FIGURE 2 Reactivity of y-FA and s-FAs towards acetylcholine and ROK-inhibitor, Y27632. (A–F) Effect of cumulatively increasing concentrations of acetylcholine (A, B) and the ROK-inhibitor, Y27632 (E, F) on submaximal force of y-FA and s-FAs. (C, D) Original records and statistic calculation of the effect of acetylcholine after NO-inhibition by 100 μmol/L L-NAME. Force calculated in % from contraction reached prior to application of the first concentration of the tested substances accepted as 100%. Submaximal force was elicited by 0.3 μmol/L U46619

TABLE 2 Relaxation induced by acetylcholine, ROK-inhibitor, Y27632 and NO donor, DEA-NONOate in y- and s-FAs

| Treatment                     | $F_{\text{max}}$ (%) | $-\log[pD_2]$ | $n$ |
|-------------------------------|----------------------|---------------|-----|
| Acetylcholine_Young (controls) | 63.2 ± 6.2           | 7.2 ± 0.09    | 8   |
| Acetylcholine_Senescent (controls) | 44.2 ± 4.4$^3$  | 6.9 ± 0.1$^{n.s.}$ | 8   |
| Acetylcholine_Young (L-NAME)   | 22.1 ± 1.0           | n. a.         | 6   |
| Acetylcholine_Senescent (L-NAME) | 9.1 ± 1.4           | n. a.         | 8   |
| Y27632_Young                  | 62.1 ± 3.3           | 5.3 ± 0.05    | 7   |
| Y27632_Senescent              | 59.6 ± 2.2           | 5.2 ± 0.03$^{n.s.}$ | 9   |
| DEA-NONOate_Young             | 63.2 ± 1.1           | 4.4 ± 0.1     | 4   |
| DEA-NONOate_Senescent         | 71.8 ± 5.6           | 5.6 ± 0.1$^{**}$ | 5   |

Note: n.s.: $p = 0.3648$: $-\log[pD_2]$ Acetylcholine_Senescent (controls) versus Acetylcholine_Young (controls); two-way ANOVA.

n.s.: $p = 0.9019$: $-\log[pD_2]$ Y27632_Senescent versus Y27632_Young; two-way ANOVA. n. a.: Note, due to non-sigmoidal pattern of concentration response relationships in these cases, the $pD_2$ values have not been calculated.

$p = 0.0108$: $-\log[pD_2]$ Acetylcholine_Senescent versus Acetylcholine_Young; two-way ANOVA.

$^3p < 0.05$: $-\log[pD_2]$ DEA-NONOate_Senescent versus DEA-NONOate_Young; two-way ANOVA.
previously that MLCP activity is regulated by inhibitory phosphorylation of MYPT1 at residue T696 and to lesser extent at T853.21,22 In senescent FAs, the T853 immunoreactive signal was reduced by approx. 25% (Suppl. Fig. 4) under basal conditions (PSS, no stimulation) and as expected, treatment with 3 μmol/L Y27632 abolished phosphorylation at T853 completely (Figure 3A,D). In controls from both age groups, the immunoreactive signals of pT696 were nearly equal. While in y-FAs Y27632 had no effect on MYPT1-T696 phosphorylation, treatment with the ROK-inhibitor reduced MYPT1-T696 phosphorylation by approx. 25% in s-FAs (Figure 3A,E).

We further tested whether observed increase in MLCP20 phosphorylation might be due to reduced PKG activity with advanced age. To test this hypothesis, we explored phosphorylation of MYPT1-S668. This MYPT1 phospho-site has been shown to be predominantly phosphorylated by PKG and may serve as a marker for the activity of this enzyme.23 We found that MYPT1-pS668 immunoreactivity was similar in both age groups and treatment with ROK inhibitor had no effect on this phosphorylation (Figure 3A,F). In addition, we found that expression levels of total MYPT1, normalized to actin, were not altered by senescence (Figure 3A,G).

We also tested whether the observed distinct reactivity of Y27632 in y- and s-FAs might relate to differences in endothelial function. For that, we determined MLC20-S19 in mechanically de-endothelialized y- and s-FAs under control conditions and after treatment with 3 μmol/L Y27632. De-endothelialization reduced MLC20-S19 by ~50%, but did not alter the dephosphorylating effect of Y27632 in s-FAs (Figure S1A, B). Effectiveness of the de-endothelialization has been proven by probing the blot membranes with phospho-specific antibodies against the PKG site of MYPT1, MYPT1-S668. Phosphorylation at MYPT1-S668 was
reduced by ~70% in both age groups, an extent similar to incubation with the pan-NOS-inhibitor L-NAME. (Figure S3A,E). De-endothelialization also reduced immunoreactivity of the ROK site of MYPT1, T853, but did not alter the dephosphorylating effect of Y27632 (Figure S1A,C). By contrast, mechanical rubbing reduced phosphorylation of MYPT1-T696 by ~20% supporting the notion that this phospho-site has major impact in the regulation of MLCP in smooth muscle cells (Figure S1A,C).

Interestingly, by endothelium removal, the ratio between MYPT1-total and α-actin was reduced by 50%, suggesting that MLCP is highly expressed in vascular endothelium (Figure S1F).

3.5 | In FAs, phosphorylation of RSK2 and NM-myosin was not altered by senescence

Together with the classical, Ca\(^{2+}\)-dependent way for activation of the acto-myosin complex, it has been demonstrated in a recent publication that activated p90 ribosomal S6 kinase type 2 (RSK2) is also able to phosphorylate MLC\(_{20}\) and thus to regulate myogenic contraction without significant effect on Ca\(^{2+}\) homeostasis.\(^{24}\) The same work showed that phosphorylation of RSK2 at Serine-227 (S277) increased its activity and is tightly associated with the increase in the myogenic or agonist-induced vasoconstriction. To test whether RSK2 phosphorylation is involved in increased stress-induced tone of s-FAs, we also monitored phosphorylation of the kinase at its activation site, S277, in preparations obtained from both age groups. We found no significant difference in basal RSK2-pS277 levels in both y- and s-FAs (Figure 4A,B).

Nowadays, it has been proposed that together with phosphorylation of myosin II, a small pool of membrane-associated non-muscle myosin (NM-myosin) is involved in regulation of smooth muscle contraction.\(^{25}\) These myosin molecules exist in polymerization incompetent ‘folded’ and polymerization competent ‘unfolded’ conformations, whereby the latter depends on phosphorylation at its S1943 site. To test whether in s-FAs this activation site is phosphorylated, we determined the immunoreactivity of NM-myosin-S1943 in both age groups. NM-myosin-S1943 was phosphorylated to a similar degree in both age groups (Figure 4A,C).

3.6 | Increased expression of vimentin, but not of caldesmon and desmin in s-FAs

Beside phosphorylation of myosin, other factors determine the contractility of the acto-myosin complex. One such protein is caldesmon.\(^{26}\) This actin-, myosin-, calmodulin- and tropomyosin-binding protein, discovered over four decades ago, has been shown to be able to inhibit myosin Mg-ATPase activity and thus to change the contractile state of smooth muscle cells without alteration of myosin phosphorylation. To test whether caldesmon is less abundant in s-FAs than in y-FA, which could contribute to stress-induced tone in s-FAs, we compared expression of caldesmon in FAs from both age groups. There was no difference in immunoreactive signal-intensity of caldesmon in s-FAs compared to those measured in y-FAs (Figure 4A,D), as we were only able to detect the high molecular isoform of caldesmon, h-caldesmon.

Myogenic contractility is also thought to relate to the expression pattern of the type III intermediate filament proteins desmin and vimentin. These proteins are widely expressed in striated, cardiac, and smooth muscles and have been shown to be responsible for the transduction of mechanochemical signals from the plasmalemma to the cytoskeleton. In addition, they are early markers for cell differentiation.\(^{27}\) We therefore examined whether the expression of these proteins is altered in s-FAs and found a significant increase in immunoreactivity of vimentin, but not of desmin (Figure 4A,D).

3.7 | Expression ratio of Exon24-in/out isoform of MYPT1 and sensitivity to cGMP/PKG system of y- and s-FAs

Overexpression of vimentin arrests cell differentiation, and its expression level is used as marker for undifferentiated and proliferative cells.\(^{27}\) Also typical for proliferative vascular tissues in early developmental stage or dedifferentiated smooth muscle cells in cell culture\(^{18}\) is a high expression level of the MYPT1-isoform lacking Exon24 (Exon24-out). We therefore tested the hypothesis, whether senescence changes the expression ratio of Exon24-in/out isoforms of MYPT1 in direction of higher Exon24-out expression. In y-FAs, we found an Exon24-out expression ratio of 22.8 ± 1.5% Exon24-out expression, compared to an expression of 33.5 ± 3.3% expression ratio in s-FAs (p = 0.011, Figure 5A). Expression of Exon24-out isoform of MYPT1 was validated by using urothel as positive control and Exon24-in expression by using preparations from M. detrusor, both gained from urinary bladder (Figure S2).

To ensure that the observed difference in NO sensitivity is not due to different PKG expression, we determined the expression of the enzyme in y- and s-FAs. No difference in both age groups was detected (Figure 5B). The data were in agreement with experiments showing no
difference in MYPT1-S668 (Figure 3F), a site shown to be specifically phosphorylated by PKG.

We showed earlier that a higher expression ratio of Exon24-out isoform of MYPT1 in brain vascular system is associated with higher ability of these vascular preparations to generate spontaneous SIT and respond to activation of the cGMP/PKG system. To further test whether this also applies to s-FAs, we compared in the following the contractile response of FAs from both age groups after stretch and inhibition of endogenous NO release. As described previously, stretch during the initial preparation induced increase in SIT in s-FAs, but not in y-FAs (Figure 5C). Inhibition of endogenous NO release by 100 μmol/L L-NAME induced a further increase in tone in s-FAs, but had no effect on ring preparations from y-FAs (10.4 ± 1.2 mN in s-FAs vs. 4.8 ± 0.3 mN in y-FAs; Figure 5C). In line with these findings, we also found that reactivity to NO donor (DEA-NONOate) shifted leftward in senescent FAs (Figure 5D).

In line with previous observations in non-stimulated rings from s-FAs, phosphorylation of MLC20-S19 was significantly higher compared to that detected in y-FAs (Figure 5E,F). Although in y-FAs, L-NAME had no significant effect on force, application of the compound was able to increase MLC20-S19 levels similar to those observed in L-NAME treated s-FAs (Figure 5E,F). Interestingly, the same treatment of s-FAs with L-NAME was without significant effect on MLC20-S19, supporting the view that the mechanism of the observed effect on force is distinct from phosphorylation of the regulatory light chain of myosin (Figure 5E,F). Inhibition of endogenous NO release by L-NAME reduced phosphorylation of the PKG site of MYPT1, S668, to an equal extend (Figure S3A,C) and induced a small increase on MYPT1-T853 (Figure S3A, B). Treatment with L-NAME was without effect on MYPT1-T696 (Figure S3A,D).

**FIGURE 4** Effect of senescence on phosphorylation of NM-myosin and RSK2.
Expression of α-actin, β-actin, caldesmon, vimentin and desmin. (A) Original luminograms from western blots performed with preparations from y- and s-FAs either under control conditions or treated with 3 μmol/L ROK-inhibitor, Y27632. (B, C) Statistic evaluation of phosphorylation of NM-myosin-S1943 (n = 4–5), RSK2-S277 (n = 8). (D) Statistic evaluation of α-actin (n = 6–5), β-actin (n = 6–5), caldesmon (n = 4), vimentin (n = 6–7) and desmin (n = 6–7) expression in y- and s-FAs. All data normalized to mean value of immunoreactive signals obtained from controls (non-treated) y-FAs, accepted as 100%.

**DISCUSSION**

The present work provides evidence that senescence increases stress-induced contractility of FAs is increased during senescence. The underlying mechanisms are linked with: (1) Increase in basal phosphorylation of regulatory light-chain of myosin, MLC20-S19. (2) We also report an increased expression of intermediate filament protein vimentin in s-FAs. (3) An isofrom switch in the expression ratio of Exon24-in/out isoforms in the targeting
subunit of myosin phosphatase type I, MYPT1, of s-FAs, leading to (4) an increased sensitivity of contractile myofilaments of s-FAs towards activation of the NO/cGMP/PKG system.

4.1 Contractility of the femoral artery in young age and senescence

As in other vascular beds, the contractile state of the common femoral artery is functionally dependent on the phosphorylation of the regulatory light chain of smooth muscle myosin IIa. This Ca$^{2+}$-dependent, reversible phosphorylation enables myosin-ATPase activity, facilitates interaction with actin and is a prerequisite for crossbridge cycling. Rise in FAs contractility is not solely controlled by myoplasmic Ca$^{2+}$, but rather supported by G-protein-coupled increase of Ca$^{2+}$ sensitivity by RhoA kinase (ROK) or protein kinase C (PKC). The signal cascades of both enzymes converge in the inhibition of the enzyme MLCP, followed by increased phosphorylation of MLC$_{20}$ and lead to a rise in force, an event defined as Ca$^{2+}$ sensitization.

It is well appreciated that FAs display a specific contractile pattern, as the level of basal phosphorylation is higher than in brain vessels or visceral smooth muscles. By agonist stimulation at the early phase, this involves a direct interference of MLCP activity by the ‘C-kinase-potentiated protein phosphatase-1 Inhibitor’ (CPI-17). This 17-kDa, small protein is highly abundant in rabbit FAs, and its inhibitory effect is enhanced by PKC phosphorylation at threonine-38 (T-38). In FAs, a downregulation of PKC by phenylephrine or endothelin-1 prevented Ca$^{2+}$ sensitization, as application...
of a PKC inhibitor can cause desensitization even at Ca$^{2+}$-free conditions.29 Herein, report no phosphorylation of CPI-17-T38 in y- and s-FAs at basal conditions (Figure 2A,B), supporting the view that this protein most likely is not involved in accelerated SIT. Our study is in agreement with others, reporting no phosphorylation at basal condition or depletion of Ca$^{2+}$ stores.4,32

MLCP is a heterotrimeric holoenzyme of which the catalytic activity could also be inhibited via threonine phosphorylation of its targeting subunit, MYPT1.33 MYPT1 contains multiple phosphorylation sites, as for two of them, T696 and T853 have been reported to be able to allosterically inhibit the catalytic activity of MLCP holoenzyme.22,34 While the degree of T696 of MYPT1 has been shown to directly act on MLCP activity,21 phosphorylation of MYPT1-T853 seems to play a minor role in the enzyme regulation.35 MYPT1-T853 serves as an internal ROK sensor phosphorylation, which might be used as reference for the degree of ROK activity. Herein, we report that inhibition of ROK by Y27632 relaxes y- and s-FAs to the same extent (Figure 2E,F) and completely inhibits phosphorylation of MYPT1-T853 (Figure 3A,D). Regarding other phospho-threonine ROK site, we and others report that in y-FAs, phosphorylation at T696 is not sensitive to ROK inhibition, suggesting that this site does not participate in basal and agonist-induced tone regulation4,39, present study. However, compared to young FAs, MYPT1-T696 phosphorylation in s-FAs was reduced by inhibition of ROK by Y27632 (Figure 3A,E). Such a ROK-sensitive inhibition due to T696 phosphorylation has been recently reported in brain vasculature, in situ.36 In line with this, denervated aortic and brain vascular rings (tissues expressing to a higher degree MYPT1-mRNA with Exon24 exclusion5), subjected to mechanical stretch, are able to generate spontaneous tone.36,37 Based on aforementioned findings, we hypothesize that such a remodelling of s-FAs does not alter ROK activity per se but favours a greater contribution of MYPT1-T696 phosphorylation and MLCP, respectively, in tone maintenance than other ROK-sensitive regulating mechanisms such as actin-polymerization or Ca$^{2+}$ mobilization. Thus, we hypothesize that MYPT1, containing a C-terminal leucine zipper domain, may not only be responsible for association with the PKG LZ motif, but rather also allosterically inhibits the MLCP holoenzyme and rises MLC$_{20}$ phosphorylation and tone, respectively.

4.2 Isoform switch of Exon24 MYPT1 and regulation of NO/GC/PKG sensitivity in ageing and senescence

A number of seminal studies recently revealed that the reactivity of smooth muscle to the endogenous NO/GC/PKG system could be accelerated in vitro by dimerization of leucine zipper motives present on the C-terminus of MYPT1 and the N-terminus of PKG18,38 (rev. Dippold and Fisher33). Interestingly, in some tissues like rat aorta, such a dimerization occurs even without persisting activation of PKG by cGMP, supporting the importance of this interaction for maintenance of vascular tone and reactivity.18 There is a consensus in the literature that two major isoforms of MYPT1 protein, generated by splicing, are expressed in the smooth muscle wall of all hollow organs including the vascular system.33 Typically, the walls of all gastrointestinal and urinary organs and those of the cardiovascular systems express a mixture of LZ$^+$ and LZ$^-$ MYPT1 isoforms (rev. Dippold and Fisher33). Remarkably, in some of them with high smooth muscle cell content, like urinary bladder, expression of LZ$^-$ is terminated even in fetal phase.35 With regard to the function of vascular tissue, typically a posttranscriptional modification occurs postnatally, as the more sensitive to PKG LZ$^+$ isofrom of MYPT1 is replaced by the less PKG-sensitive MYPT1-LZ$. Parallel to this process, there is an increase in mean blood pressure due to maturation.18,39 Thus, in the mature murine organism, only aorta and to some extent the vascular tissue of the brain circulatory system express predominantly the LZ$^+$ isofrom of MYPT1 and show an increased sensitivity to NO/cGMP/PKG activation, compared to systemic arteries.5,18 The predominant expression of PKG-insensitive MYPT1-LZ$^-$ seems to be not constitutive as some pathological conditions like HF, flow restriction and endotoxemia are able to promote the re-expression of the MYPT1-LZ$^+$ isofrom.11,40,41 Herein, we report that compared to y-FAs, s-FAs express to the greater extent Exon24-out mRNA and this is probably the causal link for the observed augmented sensitivity of the smooth muscle to inhibition of NO release or exogenous NO application. In contrast to endotoxemic conditions, which also lead to a similar increase in Exon24-out mRNA (rev. in Reho et al.11), in our senescence model, neither reduction of the contractile proteins such as $\alpha$-actin, caldesmon or desmin nor change in the PKG expression has been observed (Figures 4A,C and 5E,F). In line with this, the phosphorylation of MYPT1-S668, a site of MYPT1, which serves as specific PKG sensor, was also not altered by senescence (Figure 3A,F). As a result, compared to the endotoxemic model herein, a dampening of contractility in s-FAs has not been observed. Our study is in accordance with the comprehensive publication of Reho and colleagues, who reported that the specific re-expression of the MYPT1-LZ$^+$ isofrom of MYPT1 in smooth muscle accelerates sensitivity of systemic vasculature to NO
and cGMP without a dramatic change in vascular contractility.\textsuperscript{11} The same study reported that this genetic manipulation leads to decrease of the mean arterial pressure by 10 mmHg.\textsuperscript{11} All these data support the hypothesis that re-expression of MYPT1-LZ\textsuperscript{+} without loss of contractile proteins might act as internal brake against pathological increase of mean blood pressure in ageing and senescence. Thus, re-expression of the more PKG-sensitive isoform of MYPT1 might increase the NO reserve of the vascular system, leading to dampening of hydrostatic pressure. In same context, it seems to be that together with the re-expression of MYPT1-LZ\textsuperscript{+}, s-FAs undergo remodeling in another direction leading to silencing of ROK, which additionally may counteract the pathologic rise of the intrinsic tone. Herein we report that the phosphorylation of MYPT1-T853, a site whose phosphorylation degree is solely regulated by ROK and thus might serve as a marker for intrinsic activity of the enzyme, is reduced in s-FAs (Figure S4).

Similar mechanisms may also be involved in the protection of myocardial ischaemia-reperfusion injury.\textsuperscript{42} Based on observations in this and other studies, we hypothesize that this might be an important compensatory mechanism, which contributes to maintain normal blood pressure in senescence and serve as element of so-called healthy ageing.

### 4.3 Clinical context

From a clinical point of view, there is a coincidence of HFpEF and arterial stiffness. HFpEF was initially considered to be a haemodynamic disorder, characterized by hypertension, cardiac hypertrophy and diastolic dysfunction. Currently, the complexity of HFpEF is recognized, and the role of cardiac myocyte calcium handling\textsuperscript{43} and sarcomeric relaxation, energy metabolism, and mitochondrial function are acknowledged as players in addition to factors as ECM deposition and extracardiac reasons. The TOPCAT trial supported the existence of distinct clinical HFpEF phenogroups with contemporaneous stiffness of the large arteries in senescent patients.\textsuperscript{44} In our study, we showed that augmented SIT of senescent femoral arteries was associated with increase in MLC\textsubscript{20} phosphorylation and responsiveness to NO. Consequently, NO treatment could help decrease vascular tone in senescence. This in turn might be beneficial in the treatment of the subset of HFpEF patients with increased vascular stiffness. NO is currently not a therapeutic choice in the treatment of HF patients but is used as tailored therapy for HFpEF patients with increased vascular tone due to senescence in order to release symptoms or progression of HF.

### 4.4 Study limitations

Determination of mechanic stretch response of femoral artery and the biochemical analysis of regulatory phosphorylation were performed at steady state, which may not fully match the status of these vessels under dynamic conditions.

Given that ROK inhibition by Y27632 might influence Ca\textsuperscript{2+} homeostasis\textsuperscript{45,46} and thus indirectly decrease MLC\textsubscript{20}-S19 phosphorylation, our results do not exclude a potential activation of this regulatory cascade in pathological increase of spontaneous tone.

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### CONFLICT OF INTEREST

Authors declare no potential conflict of interests.

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