Effect of age on the vascular proteome in middle cerebral arteries and mesenteric resistance arteries in mice

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Abstract

Aging is associated with hypertension and brain blood flow dysregulation, which are major risk factors for cardiovascular and neurodegenerative diseases. Structural remodeling, endothelial dysfunction, or hypercontractility of resistance vessels may cause increased total peripheral resistance and hypertension. Recent studies showed that G protein- and RhoA/Rho-kinase pathways are involved in increased mean arterial pressure (MAP) and arterial tone in middle-aged mice. We aimed to characterize the age-dependent changes in the vascular proteome in normal laboratory mice using mass spectrometry and bioinformatics analyses on middle cerebral arteries and mesenteric resistance arteries from young (3 months) vs. middle-aged (14 months) mice. In total, 31 proteins were significantly affected by age whereas 172 proteins were differentially expressed by vessel type. Hierarchical clustering revealed that 207 proteins were significantly changed or clustered by age. Vitamin B6 pathway, Biosynthesis of antibiotics, Regulation of actin cytoskeleton and Endocytosis were the top enriched KEGG pathways by age. Several proteins in the RhoA/Rho-kinase pathway changed in a manner consistent with hypertension and dysregulation of cerebral perfusion. Although aging had a less profound effect than vessel type on the resistance artery proteome, regulation of actin cytoskeleton, including the RhoA/Rho-kinase pathway, is an important target for age-dependent hypertension.

1. Introduction

Aging is associated with an increase in the occurrence of arterial hypertension and brain blood flow dysregulation, which are major risk factors for cardiovascular diseases, stroke, and cognitive impairment in both sexes (Fuchs and Whelton, 2020; Hay et al., 2020; Ungvari et al., 2021). In general vascular stiffness, intima-media thickness, and lumen diameter of large conductance arteries are increased with aging (Rizzoni et al., 2019). Large artery stiffening is associated with increased systolic blood pressure and pulse pressure during aging (Lakatta, 1989, 2015), and is accompanied by increased pulsatility in the smaller downstream vessels. The Framingham Heart Study showed that mean arterial blood pressure (MAP) increases with age in men and women until the age of ~70 (Cheng et al., 2012). MAP is the product of cardiac output and the total peripheral resistance (TPR), which is governed by the diameter of the resistance arteries (300–100 μm) and arterioles (<100 μm) (Mulyani and Aalkjaer, 1990). Age-dependent increases in MAP associated with increased TPR may be the result of structural remodeling, endothelial dysfunction, or hypercontractility of resistance arteries and arterioles, all of these resulting in a reduced vessel diameter and increased TPR. Structural remodeling is a slow adaptation of the passive lumen diameter and/or media thickness to a change in hemodynamic forces or vascular tone. In small resistance arteries wall thickness, lumen diameter, and wall cross-sectional area are generally increased with aging in humans (Rizzoni et al., 2019). The media:lumen-ratio of small subcutaneous resistance arteries is positively correlated with age in

Abbreviations: DTT, dithiothreitol; IAA, iodoacetamide; LC-MS/MS, liquid chromatography-coupled tandem mass spectrometry; Lys-C, lysyl endopeptidase; MAP, mean arterial pressure; MCA, middle cerebral artery; MRA, mesenteric resistance artery; NO, nitric oxide; SDC, sodium deoxycholate; SLS, sodium lauroyl sarcosinate; TFA, trifluoroacetic acid; TPR, total peripheral resistance; VSMC, vascular smooth muscle cell.

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normotensives, and this correlation is more steep in hypertensive patients (Bruno et al., 2017). Endothelial function in humans, measured as the maximal blood flow response to arterial infusion of acetylcholine, is significantly reduced by age in both normotensive and hypertensive subjects, with the hypertensives having a lower total blood flow but a similar age-dependency (Taddei et al., 1997). Age-dependent endothelial dysfunction can be caused by reduced ability of the endothelium to release vasodilators such as nitric oxide (NO), prostacyclin and endothelium-dependent hyperpolarization, but also by increased production of endothelial vasoconstrictor prostaglandins (Vanhoutte et al., 2017). Increased smooth muscle cell contractility may result from a shift in the balance from NO- to Endothelin-1 release from the endothelium (Vanhoutte et al., 2017), or an increased activity of the vasoconstrictor hormone Angiotensin II in vascular smooth muscle cells (VSMC) (DuPont et al., 2016; McCurley et al., 2013). This may be exaggerated by an age-dependent decrease in K⁺ channel expression causing depolarization of the VSMCs (Toro et al., 2002). On the other hand, in advanced age associated with VSMC senescence, the basal myogenic tone and noradrenergic contractility in resistance arteries seem to decline (Gros et al., 2002; Kang et al., 2009; Seawright et al., 2018; Springs et al., 2015; Toth et al., 2017). A change from a contractile to a synthetic VSMC phenotype may thus contribute to age-dependent decline in contractility (Chi et al., 2019; Muller-Delp et al., 2018).

Fischer-344 rats are often used for aging studies, but they do not display age-dependent hypertension and are insensitive to salt-dependent hypertension. In contrast, Sprague-Dawley rats display some degree of age-dependent blood-pressure increase (Bunag and Teravainen, 1991; Reverte et al., 2013). Mice are in general considered very good mammalian models for aging research in humans due to: 1) a short life-span, 2) the feasibility of performing genetic manipulations, 3) mice share many of the same age-related phenotypes and diseases found in human subjects, 4) and they share the genetic loci influencing lifespan and health-span with humans (Ackert-Bicknell et al., 2015; Folgueras et al., 2018; Vanhooren and Libert, 2013). Recently, regular C57BL/6 laboratory mice have been used for studying age-related increases in blood pressure involving increased activation of pro-contractile Gα11 and G₁₂-proteins in VSMCs (Wirth et al., 2016). We recently showed that the vascular wall undergoes a hypertrophic response by age in mouse mesenteric resistance arteries (Mikkelsen et al., 2016). We also found that the myogenic tone as well as the noradrenergic tone was increased in mature adult and middle aged mice compared to young ones (Björling et al., 2018; Mikkelsen et al., 2016). The increased myogenic tone was associated with an age-dependent increase in Rho-kinase 2 (ROCK2) mRNA expression, and an increased sensitivity to ROCK2-specific inhibition of myogenic tone (Björling et al., 2018). Thus, middle-aged mice may develop hypertension due to increased activity of the RhoA/ROCK pathway and upstream Gα₁₁ and G12-coupled receptors.

Physiological, biochemical and molecular biological studies have revealed detailed knowledge about the function of individual proteins and cellular signaling pathways in small arteries and arterioles. However, large-scale proteomics using advanced mass spectrometry makes it possible to characterize the identity and relative abundance of all proteins expressed in a biological sample and to achieve an “unbiased” characterization of changes occurring at the protein level in resistance arteries. Nevertheless, only scarce information has been published so far based on proteomic analyses of resistance arteries in hypertension and aging. To form the basis of future studies on new drug targets and preventive measures of complications in hypertension and aging, we therefore set out to characterize the age-dependent changes in the vascular proteome in normal laboratory mice. We employed young mice (3 months old) vs. middle-aged, but not senescent, mice (14 months old) and we compared the proteomic changes in middle cerebral arteries controlling blood flow to the cortex in both cerebral hemispheres vs. mesenteric resistance arteries involved in regulation of MAP and blood flow to the gut. Based on thorough bioinformatics analyses of our liquid chromatography-coupled tandem mass spectrometry (LC–MS/MS) data from these resistance arteries, we investigated which proteins and biological pathways form interesting candidates for future studies on age-related changes in resistance artery function and regulation of blood pressure and flow.

Thus, the primary aim of our study was to provide an overall evaluation of the impact of age on the expression pattern and relative abundance of individual proteins in mouse middle cerebral arteries and mouse mesenteric resistance arteries. A secondary aim was to analyze the significance of vascular bed on the vascular proteome in both young and middle-aged mice. Finally, we aimed to identify, through bioinformatics analyses, the most likely protein networks and pathways that may contribute to age-related changes in the vascular structure and function, such as in arterial hypertension and brain blood flow dysregulation leading to increased risk of cardiovascular and cerebrovascular events in the elderly.

2. Materials and methods

2.1. Animals and tissue isolation

Experiments were conducted at the National Cerebral and Cardiovascular Center (NCVC) Research Institute, Osaka, Japan with approval of the Animal Experiment Review Committee (No. 19030). Male C57BL/6 J mice were purchased at 2½ vs. 8 months of age (N = 8 vs. N = 7, Japan SLC, Hamamatsu, Japan) and group housed in the NCVC Animal Facility with ad libitum access to water and a normal chow diet until the time of experimentation (3 vs. 14 months of age). Following induction of deep anaesthesia (3–4 % isoflurane, Wako, Osaka, Japan) the mice were killed by cervical dislocation and the brain and gastrointestinal tract including the mesenteric vascular bed were carefully removed by dissection and stored in cold PBS solution for subsequent blunt dissection of the middle cerebral arteries (MCA) and small mesenteric resistance artery (MRA) 3rd order branches under a stereomicroscope. We collected ~1 mg fresh MCA tissue and ~35 mg fresh MRA tissue (wet weight) from young and middle-aged mice, respectively. The tissues were pooled into four groups: “Young” MCA (YMCA, N = 8); “Aged” MCA (AMCA, N = 7); “Young” MRA (YMRA, N = 8); and “Aged” MRA (AMRA, N = 7), and kept at −80 °C until used.

2.2. Materials

Sodium deoxycholate (SDC), sodium lauryl sarcosinate (SLS), dithiothreitol (DTT), iodoacetamide (IAA), l-lysyl endopeptidase (Lys-C), ethyl acetate, acetonitrile, acetic acid, and trifluoroacetic acid (TFA) were purchased from Wako (Osaka, Japan). Modified trypsin was from Promega (Madison, MA). C18 Empore products were purchased from 3 M (St. Paul, MN). Water was obtained from a Millipore Milli-Q system (Bedford, MA).

2.3. Sample preparation

Protein extraction was performed according to our previous reports (Masuda et al., 2008; Wakabayashi et al., 2014). Blood vessels were pulverized under frozen conditions at −80 °C using a Shake Master Neo instrument (BMS, Tokyo, Japan), suspended in PBS buffer (12 mM SDC, 12 mM SLS in 100 mM Tris-HCl, pH 9.0), incubated on a heating block at 95 °C for 10 min, and sonicated for 10 min in ice-cold water. Recovered proteins were applied to reduction and alkylation with 10 mM DTT and 50 mM IAA, respectively. The sample solutions were 5-fold diluted with 50 mM ammonium bicarbonate and digested with Lys-C for 3 h, followed by overnight trypsin digestion. After digestion, equal volume of ethyl acetate was added and then acidified with 0.5 % TFA (final concentration). The mixture was agitated for 2 min and centrifuged at 15, 800 g for 2 min to completely separate aqueous and organic phases. The aqueous phase was collected and desalted with C18-StageTips.
Peptides were separated on an ODYSSEY column mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, (1.6 μm C18, 120 Å, 75 μm ID, 25 cm) from IonOpticks. The injection volume was 2 μL and the flow rate was 400 nL/min. The mobile phases consisted of (A) 0.1 % formic acid and (B) 0.1 % formic acid in 80 % acetonitrile. A four-step linear gradient of 4–34 % B in 90 min, 34–48 % B in 15 min, 48–90 % B in 5 min, and 90 % B for 10 min was employed. The mass scan range was 100–1700 m/z, and the ion mobility was scanned from 0.6 to 1.6 Vs/cm. The overall scan cycle of 1.16 s included a single MS scan and ten PASEF MS/MS scans (Meier et al., 2018). Low abundance precursor ions below a target intensity value of 20000 counts were repeatedly selected for PASEF-MS/MS. Active exclusion time was set to 0.4 s.

2.4. NanoLC–MS system

NanoLC–MS/MS analyses were conducted by using a timsTOF Pro mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, Bremen, Germany). Peptides were separated on an ODYSSEY column mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, Bremen, Germany). Peptides were separated on an ODYSSEY column mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, Bremen, Germany). Peptides were separated on an ODYSSEY column mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, Bremen, Germany). Peptides were separated on an ODYSSEY column mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, Bremen, Germany). Peptides were separated on an ODYSSEY column mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, Bremen, Germany). Peptides were separated on an ODYSSEY column mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, Bremen, Germany). Peptides were separated on an ODYSSEY column mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, Bremen, Germany). Peptides were separated on an ODYSSEY column mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, Bremen, Germany). Peptides were separated on an ODYSSEY column mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, Bremen, Germany). Peptides were separated on an ODYSSEY column mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, Bremen, Germany). Peptides were separated on an ODYSSEY column mass spectrometer equipped with a nanoElute HPLC (Bruker Daltonics, Bremen, Germany).

2.5. Mass spectrometry data analysis

The raw files were analyzed with PEAKS studio X+ software (Bioinformatics solutions Inc., Waterloo, ON, CA). Peptides and proteins were identified against UniprotKB/Swiss-prot release 2019_05 with a precursor mass tolerance of 20 ppm, a fragment ion mass tolerance of 0.1 Da and strict trypsin specificity (Olsen et al., 2004) allowing for up to 2 missed cleavages. Cysteine carbamidomethylation was set as a fixed modification, and methionine oxidation and protein N-terminal acetylation were allowed as a variable modification. The peptide score (-10lgP) was derived from the p-value that indicates the statistical significance of the peptide-precursor spectrum match, and the protein score (-10lgP) was calculated as the weighted sum of the -10lgP scores of the protein’s supporting peptides. After removing any redundant peptides, the supporting peptides were sorted by -10lgP scores in descending order, and a kth ranked peptide contributed to the weighted sum with a weight of 1/k. Proteins with a -10lgP value of more than 20 were considered to be identified. Label-free quantitation was performed by the PEAKS Q module. Relative protein abundance was calculated based on the ratio of the peptide peak intensities, which were calculated based on the features detected from raw LC/MS data. For differential expression analysis, the proteins quantified in each sample were used. The MS raw data and analysis files have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the jPOST partner repository (Okuda et al., 2017) (https://jpostdb.org) with the data set identifier PXD024070.

2.6. Bioinformatic analyses

The two-by-two group design with N = 7–8 mice in each group allowed us to evaluate the results as the general effect of age in two vessel types, and the general effect of vessel type at two different ages. Data on protein abundance per group (YMCA, AMCA, YMRA, AMRA), was log2 transformed and analyzed with the limma package for R (Smyth, 2004). Briefly, an empirical Bayes method was applied to calculate a moderated t statistic for differential expression for each protein by performing a linear model fit on the data. This was followed by an empirical Bayes step to moderate the standard errors of the estimated log fold changes, and produce more stable estimates. Statistical comparisons were made between ‘Middle-aged’ versus ‘Young’ groups, and between MCA versus MRA groups. Significance levels and fold
Table 1

| Middle-aged vs. Young | Protein name (UNIPROT ID) | Log2 (FC) | P-value | Cellular function |
|-----------------------|---------------------------|-----------|---------|-------------------|
| Upregulated           |                           |           |         |                   |
| Wnt-10a (W10A)        | 3.653                     | 0.03084   |         | Canonical Wnt/ beta-catenin signaling pathway |
| Unconventional myosin-Vb (MYOSB) | 2.615 | 0.0021 |         | Involved in vesicular trafficking |
| Signal peptide complex subunit 2 (SPC2) | 2.614 | 0.0450 |         | Part of microsomal signal peptide complex |
| Zinc finger CCHC domain-containing protein 3 (ZCHC3) | 2.116 | 0.0389 |         | Involved in innate immune response to DNA and RNA viruses |
| Microfilament-associated protein 2 (MFAP2) | 1.984 | 0.0046 |         | Component of the elastin-associated microfibrils |
| Excitatory amino acid transporter 2 (EAAT2) | 1.852 | 0.0492 |         | Sodium-dependent, high-affinity amino acid transporter |
| Ig kappa chain V-VI region NQ2-6.1 (KV6AB) | 1.747 | 0.0369 |         | Adaptive immune response |
| Keratin, type I cytoskeletal 42 (KIC42) | 1.568 | 0.0326 |         | Structural molecule activity |
| Broad substrate specificity ATP binding cassette transporter 2 (ABC2G2) | 1.509 | 0.0331 |         | Amide transport activity (efflux transporter) |
| 60S ribosomal protein L13 (RL13) | 1.422 | 0.0295 |         | RNA binding; structural constituent of ribosome |

| Downregulated | Protein name (UNIPROT ID) | Log2 (FC) | P-value | Cellular function |
|---------------|---------------------------|-----------|---------|-------------------|
| Aldehyde oxidase 1/2 (AOXA/AOXB) | −4.696 | 0.0002 |         | Oxidase with broad substrate specificity |
| Laminin subunit alpha-3 (LAMA3) | −2.639 | 0.0078 |         | Extracellular matrix structural constituent |
| Myosin phosphatase Rho-interacting protein (M-RIP) | −2.419 | 0.0393 |         | Actin filament binding |
| EGF-containing fibulin-like extracellular matrix protein 2 (FBLN4) | −2.129 | 0.0482 |         | Calcium ion binding, elastic fiber constituent |
| Cytochrome c oxidase subunit 5B, mitochondrial (COX5B) | −1.879 | 0.0061 |         | Cytochrome-c oxidase activity |
| Histone H1.1 (H11) | −1.869 | 0.0457 |         | Double-stranded DNA binding |
| Tensin-2 (TNS2) | −1.789 | 0.0289 |         | Protein tyrosine phosphatase activity |
| ADP-ribosylation factor 6 (ARF6) | −1.751 | 0.0354 |         | GDP-binding protein involved in protein trafficking |
| ATPase family AAA domain-containing protein 9 (ATAD3) | −1.684 | 0.0398 |         | Essential for mitochondrial network organization, mitochondrial metabolism and cell growth |
| Tubulin β-1 chain (TUBB1) | −1.494 | 0.0339 |         | Major constituent of microtubules; Microtubule |

Table 1 (continued)

| Middle-aged vs. Young | Protein name (UNIPROT ID) | Log2 (FC) | P-value | Cellular function |
|-----------------------|---------------------------|-----------|---------|-------------------|
| cytoskeleton organization |

change (log2FC) values per protein on each comparison were visualized by volcano plots, while comparisons of protein results using proportional Venn diagrams were done with DeepVenn (Hulsen et al., 2005) (https://www.deepvenn.com/). Expression levels for the quantified proteins were clustered with Cluster 3.0 (de Hoon et al., 2004), using Spearman Rank Correlation as similarity metric and Centroid Linkage as the clustering method. The clusters’ tree and the heatmap for the expression levels were plotted and visualized with Java Treeview (v1.1.6r4) (Saldanha, 2004).

2.7. Functional analyses

The software STRING v11 (Szklarczyk et al., 2019) was employed to construct protein-protein interaction networks of selected proteins from the clustering analysis. In addition, differentially expressed proteins and proteins displaying an age-dependent clustering were evaluated through an overrepresentation analysis using the DAVID database (Huang et al., 2009). The Mus musculus protein-coding genome was selected as background. The goal of the latter step was to determine the top KEGG enriched pathways. In addition, manual annotation of selected proteins was performed using UNIPROT, MatrisomeDB, and PUBMED database searches.

3. Results

In total, 3015 proteins were uniquely identified in the study, and in total 1833 proteins were quantified in all four groups. Fig. 1A and B show the volcano plots based on the statistical analyses of fold change of expression in middle-aged versus young mice, and the differential expression in MCA vs. MRA vessels. The Middle-aged:Young comparison yielded 31 proteins with a more than two-fold change in expression level (numerical Log2FC exceeding ±1.0), and a P-value less than 0.05, while the MCA:MRA comparison yielded 172 proteins that were more than two-fold differentially expressed at the 5% significance level. None of these proteins were significant in both comparisons, as seen by the absence of overlap in the proportional Venn diagram in Fig. 1C. Overall, there was thus a larger effect of vascular bed over age on the proteome in middle cerebral arteries and small mesenteric resistance arteries in mice.

In Table 1 is shown a list of the ten most up- and down-regulated proteins by age. The functions of the significantly upregulated proteins vary from intracellular signaling/trafficking, membrane transporters, structural constituent/activity, to innate/adaptive immune responses. The functions of the significantly downregulated proteins vary from intracellular oxidase activity and energy metabolism, extracellular matrix and intracellular cytoskeletal structural components, to GTP or DNA binding proteins. As seen from a vasomotor function and vascular structure perspective, the most important findings are the upregulation of canonical Wnt signaling (Wnt10A), vesicular trafficking (myosin-Vb) and elastin-related extracellular matrix molecules (microfibrillar-associated protein 2). Among the significantly downregulated proteins the ones involved in extracellular laminin and elastin matrix fiber structure (Laminin-alpha3; EGF-containing fibulin-like extracellular matrix protein 2/Fibulin-4) and intracellular actin cytoskeletal structure (Myosin phosphatase Rho-interacting protein, M-RIP/p116RIP) are the most important from a vascular structure and function point of view.

In Table 2 is shown a list of the ten proteins with highest and lowest differential expression in MCA vs. MRA vessels, where the highest differential expression indicates proteins with highest expression in MCA...
and vice versa. The most important findings are that the Na/K-ATPase \( \alpha_2 \) subunit is highly expressed in MCA vs. MRA, whereas the Na/K-ATPase \( \alpha_1 \) subunit has a significant low expression in MCA compared to MRA vessels. The GLUT1 glucose transporter (Solute carrier family 2, facilitated glucose transporter member 1 (GLUT1)) had low expression in MCA compared to MRA. The GTP-binding protein/GTPase Rab-1A regulating intracellular trafficking was highly expressed in MCA vs. MRA, whereas the aquaporin-1 water channel had significantly lower expression in the MCA compared to MRA. Similarly, the GLUT1 glucose transporter (Solute carrier family 2, facilitated glucose transporter member 1) was not surprisingly highly expressed in MCA vs. MRA, whereas the aquaporin-1 water channel had significantly lower expression in the MCA compared to MRA.

As our primary objective was to investigate age-dependent effects on the proteome, we performed hierarchical clustering of the data set of 1833 proteins quantified in all 4 vessel groups as shown in the cluster tree and heatmap in Fig. 2A. With 4 levels of branching points in the tree, a total of 2 \( ^2 \) clusters could be formed. Out of these 32 possible clusters we visually identified two clusters (subcluster 2 and cluster 4) with a predominantly low expression in the middle-aged vs. young arteries, as shown by green color in AMCA and AMRA vs. red color in the young arteries (as shown in Fig. 2B). Table 3 shows proteins in the network with >3 protein-protein interactions and with annotation of their molecular function, participation in biological process, and relevance to aging, hypertension or cerebrovascular function.

| **MCA vs. MRA** | **Protein name (UNIPROT ID)** | **Log2 (FC)** | **P-value** | **Cellular function** |
|----------------|--------------------------------|---------------|-------------|-----------------------|
| **Highest expression** | Sodium/potassium-transporting ATPase subunit \( \alpha_2 \) (AT1A2) | 3.494 | 0.0001 | ATPase-coupled cation transmembrane transporter activity |
| | Serine protease HTRA1 (HTRA1) | 3.634 | 0.0042 | Serine protease activity to e.g. extracellular matrix proteins |
| | Solute carrier family 2, facilitated glucose transporter member 1 (GLUT1) | 3.325 | 0.0057 | Glucose transmembrane transporter activity |
| | Ras-related protein Rab-1A (RAB1A) | 2.959 | 0.0003 | GTPase activity; regulator of intracellular membrane trafficking |
| | Nuclear receptor corepressor 1 (NCO1) | 2.726 | 0.0403 | Mediates transcriptional repression by certain nuclear receptors |
| | Trinucleotide repeat-containing gene 18 protein (TNC18) | 2.643 | 0.0025 | Enables chromatin binding |
| | 4F2 cell surface antigen heavy chain (4F2) | 2.457 | 0.0027 | Aromatic amino acid transmembrane transporter activity |
| | Histone H2A.Z/V (H2AZ) | 2.429 | 0.0021 | Chromatin DNA binding |
| | Platelet factor 4 (PLF4) | 2.417 | 0.0020 | Immune response; hemostasis |
| | Liprin-beta-2 (LIPB2) | 2.330 | 0.0013 | Identical protein binding; focal adhesions function |
| **Lowest expression** | Zeta-sarcoglycan (SGCZ) | -3.156 | 0.0108 | Transmembrane protein; component of the sarcoglycan complex |
| | Mmp14 (MIME) | -2.905 | 0.0021 | Growth factor activity |
| | Asparagine (ASP) | -2.813 | 0.0018 | Collagen and calcium ion binding; negative regulation of TGFp receptor signaling |
| | Lumican (LUM) | -2.785 | 0.0009 | Collagen binding protein involved in collagen fibril organization |
| | Parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R) | -2.700 | 0.0025 | G protein-coupled peptide receptor activity |
| | Sodium/potassium-transporting ATPase subunit \( \alpha_1 \) (AT1A1) | -2.665 | 0.0006 | ATPase-coupled cation transmembrane transporter activity |
| | Glutathione S-transferase Mu 1 (GSTM1) | -2.482 | 0.0007 | Glutathione transferase activity |
| | Aquaporin-1 (AQP1) | -2.350 | 0.0015 | Transmembrane water transport activity |
| | Periostin (POSTN) | -2.261 | 0.0067 | Cell adhesion molecule-binding protein |
| | Parafibromin (PTMs) | -2.245 | 0.0036 | Zinc ion binding; Immune system process |

Proteins with zero interactions from subcluster 2, if they were significantly downregulated by age, overall, the proteins are involved in: protein biosynthesis/translation (ABCF1, Rpf6, Eif2s1, AIMP1); extracellular matrix component (laminin alpha-3, dermatopontin); regulation of actin cytoskeleton (M-RIP/p116Rip); innate immune response (ATAD3); and mitochondrial ATP synthesis (COX5B). The most relevant clustered proteins in relation to vascular function, structure and diseases are: Eif2s1 (ER stress, systemic and pulmonary hypertension); M-RIP/p116Rip (negative regulation of myosin-light-chain-phosphatase activity); and COX5B (lower expression in genetically hypertensive rats).

For cluster 4 (164 proteins) the STRING analysis resulted in a large network consisting of 82 proteins with 1–10 protein-protein interactions as shown in Fig. 3B. Table 4 shows proteins in the network with >3 protein-protein interactions and with annotation of their function and relevance (as for Table 3). Proteins with 0–2 interactions were also included in Table 4 if they were significantly downregulated by age. Overall, the clustered proteins in Table 4 are involved in: mRNA turnover/transcriptional regulation (RS6, HSP7C, RS4X, ABCE1, RNPS1, XPO1/Crm1, FH1L2, H11); protein biosynthesis/translation (RSSA, RLA2, Eif3E, EIF3C, EIF3 M); intracellular transport/trafficking (AP2A2, ENPL/Grp94, SNAA/alpha-SNAP, Arf6); actin or microtubule cytoskeletal organization (ARP2C2, MAREI, ROCK1, TBB1); cell cycle control (LIGAM, TNS2); extracellular matrix organization (Ecm2, FBLN4); and oxidase/reductase activity (AOX/AOXB, PTGR2). The most relevant clustered proteins in relation to vascular function, structure and diseases are: RS6 (phenotypic shift in VSMCs); HSP7C (inhibits JNK signaling in ECs, protects against ischemic stroke); RSSA (pulmonary hypertension, remodeling); ARP2C2 (VSMC migration); Eif3 M (hypertensive remodeling); ENPL/Grp94 (ER stress, type 2 diabetes); MAREI (gap junction trafficking); XPO1/Crm1 (nuclear export in ECs and VSMCs); SNAA/alpha-SNAP (Orai1 calcium-store release channels); AOX/AOXB (vascular relaxation); FH1L2 (cardiac hypertrophy, hypertensive nephropathy); Arf6 (angiogenesis, VSMC proliferation and migration); ROCK1 (cardiac and vascular hypertrophic remodeling, pulmonary hypertension, Alzheimer’s disease, heart failure); and FBLN4 (systolic hypertension, arterial stiffness, thoracic aorta aneurysm).

Next, we used the functional annotation database DAVID to identify the top enriched biological (KEGG) pathways using data from the list of significantly changed proteins by age, as well as the proteins that were
clustered in an age-dependent manner. Using the list of significantly up- or downregulated proteins by age (31 proteins) we identified Vitamin B6 (pyroxidine) metabolism as the only significantly enriched pathway (Fig. 4). This enrichment was the result of a potent and highly significant down-regulation of two isoforms of the aldehyde oxidase (AOXA and AOXB), which catalyzes the conversion of pyroxidal to 4-pyroxidate leading to excretion from the body or to further conversion to succinate semialdehyde and into the butanoate metabolism pathway (Fig. 4). This result is interesting in relation to Vitamin B6 deficiency and cerebral and cardiovascular diseases.

Using the list of significantly changed proteins by age plus the proteins with an age-dependent clustering (see Fig. 2B and C) we analyzed in total 207 uniquely identified proteins. The 17 significantly enriched KEGG pathways are shown in Table 5 with an indication of the % occurrence of proteins from the dataset and sorted by increasing P-value. It is seen that three pathways stand out as being highly significantly enriched (P < 0.01) and with an occurrence >5% in the data set. The most significant pathway Biosynthesis of antibiotics contained 13 proteins with metabolic/enzymatic activity from our input list. However, this pathway enrichment was not considered to be of direct relevance for vascular structure and function. The two other pathways Regulation of actin cytoskeleton (Fig. 5A) and Endocytosis (Fig. 5B) are relevant for vascular structure and function in aging. In Fig. 5A the red dots indicate where the age-affected proteins play a role in regulation of actin cytoskeleton. We see an involvement at the level of interaction with external hormonal and mechanical stimuli (G-proteins, integrins), in regulation of myosin light chain and contractile function (PIX, PAK, ROCK, MLCP, MLC), in formation of filopodia and lamellipodia in cell motility (PIR121, Arp2/3, F-actin), in adherens junctions (IQGAP), and in stress fiber formation and cell stiffness (F-actin). Regulation of the actin cytoskeleton is therefore considered to be of major significance for the role of aging in vascular structure and function. In the Endocytosis pathway shown in Fig. 5B enriched protein occurrence was seen at the level of the clathrin coated pits and caveolae (ARF6, E3 ligase, AP-2, caveolin), the early endosomal pathway (Arp2/3, HSC-70, SWIP, EHD3, ARF6), the late endosome (SNX1/2, SNX5), and as scaffold in controlling the GDP/GTP-ratio (Arf). Endocytosis is thus considered to be important for cellular responses in vascular aging.

Our final DAVID database analysis employed the data set of 172 proteins with a significant differential expression pattern in MCA vs. MRA vessels, and this analysis yielded in total 16 significantly enriched KEGG pathways as shown in Table 6. When selected by level of significance (P < 0.01) and >5% occurrence, the 7 pathways that stand out are: Protein digestion and absorption; Oxidative phosphorylation; Focal adhesion; Parkinson’s disease; Non-alcoholic fatty liver disease (NAFLD); Huntington’s disease; and Alzheimer’s disease. Here, the focal adhesion pathway linking extracellular matrix mecano-biology to integrin signaling in the cytoplasm is considered as the most important enriched pathway for vascular structure and function.
Aging is one of the most important risk factors in many cardiovascular and cerebral human diseases with a large personal and societal impact. Since mouse models are often used as transgenic and/or disease models, we set out to study the effect of aging on the proteome in small resistance arteries critically involved in blood pressure control and regulation of blood flow to the brain and the gut. An added benefit of using mouse models is that they age within a much shorter time span than any other conventional rodent or porcine animal model, thus reducing the cost and increasing the feasibility of aging studies using mice.

Our study was designed to test both the effect of age and the effect of vessel type on the proteome expressed in middle cerebral arteries vs. mesenteric resistance arteries from mice aged 3 months (“young”) vs. 14 months (“middle-aged”). The first finding of the study was that out of 1833 proteins that were quantified in all four groups of vessels, only 31 proteins were significantly affected by age whereas 172 proteins were significantly different in expression when comparing the middle cerebral arteries and the mesenteric resistance arteries (Fig. 1A–C). Thus, the effect of vessel type on the proteome was 4–5 times larger than the effect of age. However, hierarchical clustering of the data revealed that two discrete sets of proteins clustered due to a lower expression in the middle-aged arteries vs. young (Fig. 2A–C). The two clusters were further analyzed by STRING analyses giving rise to two distinct protein-protein interaction networks (Fig. 3A and B). Ranked by the number of protein-protein interactions and the degree to which they were significantly downregulated in middle-aged arteries, we annotated the biological functions and evaluated the physiological importance of individual proteins in the two clusters (Tables 3 and 4). In subcluster 2...

Fig. 3. A: STRING database analysis showing network of known protein-protein interactions in Subcluster 2 containing 30 proteins. For clarity, only proteins with interactions at medium confidence level are shown. See also Table 3. B: STRING database analysis showing network of known protein-protein interactions in Cluster 4 containing 164 proteins. For clarity, only proteins with interactions at high confidence level are shown. See also Table 4.

4. Discussion

Aging is one of the most important risk factors in many cardiovascular and cerebral human diseases with a large personal and societal impact. Since mouse models are often used as transgenic and/or disease models, we set out to study the effect of aging on the proteome in small resistance arteries critically involved in blood pressure control and regulation of blood flow to the brain and the gut. An added benefit of using mouse models is that they age within a much shorter time span than any other conventional rodent or porcine animal model, thus reducing the cost and increasing the feasibility of aging studies using mice.
Age-dependent hierarchical clustering (subcluster 2) proteins by number of protein-protein interactions and known functions. A subset of the proteins was significantly down-regulated by aging in the present study.

| Protein name                                      | Protein-Protein interactions (STRING) | Molecular function (UniProt) | Biological Process (UniProt) | Relevance to aging, hypertension or cerebrovascular function (PubMed) |
|---------------------------------------------------|---------------------------------------|------------------------------|-------------------------------|---------------------------------------------------------------------|
| ATP-binding cassette sub-family F member 1 (ABCF1) | 3                                     | mRNA translation initiation  | Positive regulation of translation | Required for normal embryonic development (Wilcox et al., 2017). Involved in biological aging of the myocardium (Yu et al., 2015). |
| 60S ribosomal protein L6 (Rp6)                    | 3                                     | mRNA/RNA binding, Ribonucleoprotein, Ribosomal protein | Cytoplasmic translation, ribosomal large subunit assembly | Down-regulated in cerebral cortex of mice deficient in Fmr1 (fragile X mental retardation protein) (Xu et al., 2018). |
| Eukaryotic translation initiation factor 2 subunit 1 (Eif2s1) | 2                                     | Translation initiation factor, RNA binding | Protein biosynthesis, Translation regulation | Translational control in the brain (Sossin and Goto Mattrai, 2019). Vascular cell ER stress and remodeling in systemic and pulmonary hypertension, diabetes (Di Pietro et al., 2017; Choi et al., 2016a, b; Zhang et al., 2020a, b; Wang et al., 2015; Guo et al., 2019). |
| Aminoacyl tRNA synthase complex-interacting multifunctional protein 1 (AIMP1) | 2                                     | Cytokine, tRNA-binding       | Angiogenesis, Apoptosis, Inflammatory response, Protein biosynthesis | Possible association with AD pathogenesis (Jang et al., 2017). |
| Myosin phosphatase Rho-interacting protein (M-RIP, synonym p116<sup>αβ</sup>) | 1                                     | Actin filament binding       | Negative regulation of myosin-light-chain-phosphatase activity | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −2.42; P = 0.039). (Present study). M-RIP/p116<sup>αβ</sup> Targets myosin phosphatase to stress fibers to regulate myosin light chain phosphorylation in vascular smooth muscle cells (Koga and Ikebe, 2005). Insulin-stimulated myosin phosphatase Rho-interacting proteins signaling is impaired in diabetic Goto-Kakizaki vascular smooth muscle cells (Lee et al., 2012). Depletion of M-RIP/p116<sup>αβ</sup> enhances di-phosphorylation of MLCK, and a reduction of p116<sup>αβ</sup> expression in asthmatic patients may explain the hypercontractile state of airway SMCs in asthma (Komatsu et al., 2019). |
| Laminin subunit alpha-3 (LAMA3)                   | 0                                     | Extracellular matrix structural constituent, integrin binding | Cell adhesion, signal transduction, differentiation | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −2.64; P = 0.009). (Present study). Abnormal development of glomerular endothelial and mesangial cells in mice with targeted disruption of the lama3 gene (Abrass et al., 2006). LAMA3 was significantly up-regulated in preeclamptic fetal origin cells (Kim et al., 2016). |
| ATPase family AAA domain containing protein 3 (ATAD3) | 0                                     | Nucleotide-binding, ATPase activity | Antiviral innate immune response, mitochondrial organization, regulation of apoptosis and cell growth | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −2.12; P = 0.039). (Present study) |
| Dermatopontin (DPT)                               | 0                                     | Extracellular matrix structural constituent | Cell adhesion, proliferation, collagen organization | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.42; P = 0.046). (Present study). DPT augments angiogenesis and modulates the expression of TGFβ1 and integrin α3β1 in endothelial cells (Krishnaswamy et al., 2017). DPT expression was strongly down-regulated in human saphenous varicose veins compared to normal saphenous veins (Barallobre-Barreiro et al., 2016). |
| Cytochrome c oxidase subunit 5B, mitochondrial (COX5B) | 0                                     | Cytochrome-c oxidase activity | Mitochondrial ATP synthesis coupled proton transport | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.88; P = 0.006). (Present study). Left ventricular COX-Vb protein has lower expression in genetically hypertensive rats (SHR and SHR-S2P) than in WKY control rats (Kuo et al., 2015). |

(Table 3) we identified four proteins (Eif2s1, M-RIP/p116<sup>αβ</sup>, LAMA3, COX5B) with a potential significance in vascular structure and function. The Eukaryotic translation initiation factor 2 subunit 1 (Eif2s1), which is involved in regulation of protein translation, has been shown to be involved in ER stress leading to vascular remodeling in hypertension and diabetes (Choi et al., 2016a, b; Di Pietro et al., 2017; Guo et al., 2019; Wang et al., 2015; Zhang et al., 2020a). STRING analysis showed strong interactions of Eif2s1 with Aebcl and Rp6 (Fig. 3A), suggesting that this protein network could be involved in an age-dependent loss of translational control involving remodeling in resistance arteries. The myosin phosphatase Rho-interacting protein (M-RIP/p116<sup>αβ</sup>) is an actin-binding protein involved in RhoA-mediated negative regulation of myosin light chain phosphatase (MLCP) activity (Koga and Ikebe, 2005; Surks et al., 2005). Thus, M-RIP has important functions in controlling vascular smooth muscle cell contractility. M-RIP signaling was shown to be impaired in diabetic rats (Lee et al., 2012) and its expression was reduced in airway smooth muscle from asthmatic patients (Komatsu et al., 2020), leading to the suggestion that a reduced M-RIP expression reduced in airway smooth muscle from asthmatic patients (Komatsu et al., 2020), leading to the suggestion that a reduced M-RIP expression...
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Table 4
Age-dependent hierarchical clustering (Cluster 4) proteins by number of protein-protein interactions and known functions. A subset of the proteins was significantly down-regulated by aging in the present study.

| Protein name | Protein-Protein interactions (STRING) | Molecular function (UniProt) | Biological Process (UniProt) | Relevance to aging, hypertension or cerebrovascular function (PubMed or Present Study) |
|--------------|--------------------------------------|-----------------------------|-------------------------------|----------------------------------------------------------------------------------|
| 40S ribosomal protein S6 (RS6, gene name Rps6) | 10 | Structural constituent of ribosome, mRNA binding, protein kinase binding | Role in controlling cell growth and proliferation through the selective translation of particular classes of mRNA | RS6 expression is associated with a shift of the phenotype of rat coronary arteriolar vascular smooth muscle toward proliferative, non-contractile cells in aging (Müller-Delp et al., 2018). Hypo-phosphorylation of RS6 is a molecular mechanism underlying ischemic tolerance induced by either hibernation or preconditioning (Miyake et al., 2015). Protein synthesis under translational control of RS6 is reduced under tausopathic conditions in human Alzheimer’s disease brains (Koren et al., 2019). |
| Heat shock cognate 71 kDa protein (HSP7C, gene name Hspa8) | 8 | Molecular chaperone, repressor of transcriptional activation | mRNA processing, mRNA splicing, stress response, transcription, transcription regulation | Plays a protective role in the process of ischemic stroke by inhibiting the JNK signaling pathway activation in brain vascular endothelial cells (Liu et al., 2016). Functions as an accessory protein of a hyperpolarization-activated chloride channel from rat pulmonary vein myocytes (Ökamoto et al., 2019). |
| 40S ribosomal protein S4, X isoform (BS4X, gene name Rps4x) | 7 | Structural constituent of ribosome, RNA binding, RNA binding | Involved in translation, regulation of translation, and multicellular organism development | None found |
| 40S ribosomal protein SA (RSSA, gene name Rpsa) | 7 | Structural constituent of ribosome, laminin binding, laminin receptor activity | Translation, cell-cell adhesion | Increased expression in right ventricle (RV) during pulmonary hypertension and RV remodeling (Hansen et al., 2020). |
| 60S acidic ribosomal protein P2 (RLA2, gene name RpLp2) | 6 | Structural constituent of ribosome | Translational elongation | None found |
| ATP-binding cassette sub-family E member 1 (ABCE1) | 6 | ATP binding, ATPase activity, endoribonuclease inhibitor activity | Involved in regulation of mRNA turnover. Belongs to the ABC transporter superfamily, but the transporter domains are not functional. | ABCE1 was highly expressed and enriched in microvascular endothelial cells from human, rat, mouse, pig, and cow (Warren et al., 2009). |
| Actin-related protein 2/3 complex subunit 2 (ARPC2) | 6 | Actin filament binding, structural constituent of cytoskeleton | Actin filament polymerization | ARPC2 was identified as a downstream target of Nox1 activation leading to vascular smooth muscle cell migration (Chouleth et al., 2013). |
| AP-2 complex subunit alpha-2 (AP2A2) | 6 | Component of the adaptor protein complex 2 (AP-2), clathrin adaptor activity | Clathrin-dependent endocytosis, intracellular protein transport, | None found |
| Eukaryotic translation initiation factor 3 subunit E (EIF3E) | 6 | Protein N-terminus binding, translation initiation factor activity | Component of the eIF-3 complex required for initiation of protein biosynthesis | EIF3E interacts specifically with HIF-2α and induces hypoxia-independent degradation of HIF-2α (Hashimoto and Shibasaki, 2015). EIF3E silencing was suggested as a potential therapeutic strategy to improve cell survival and function after ischemic injuries (Seesen et al., 2017). Recombinant overexpression of EIF3C in human hepatocellular carcinoma cells induces enrichment of Vascular Endothelial Growth Factor signaling (Li et al., 2017). |
| Eukaryotic translation initiation factor 3 subunit C (EIF3C) | 5 | Translation initiation factor | Component of the eIF-3 complex required for initiation of protein biosynthesis | EIF3 M was found overexpressed in both hypertension and hypertensive left ventricular remodeling in humans (Pang et al., 2020). Exposure to Low-Density Lipoprotein L5 in vascular endothelial cells led to down-regulation of Endoplasm (Gpr94), ER dysfunction, and ER stress (Chen et al., 2012). Hypothermal Gpr94 expression was reduced in the offspring of High-Fat fed female rats (Nguyen et al., 2017). Intermedin (CGRP family peptide) treatment led to increased Gpr94 expression in cultured pulmonary artery smooth muscle cells under hypoxic conditions (Mas et al., 2014). 4-phenylbutyric acid treatment in mice reduced Gpr94 expression, reduced ER stress, and prevented development of hypoxia-induced pulmonary arterial hypertension (Koyama et al., 2014). ER stress induced vascular calcification in human arteries by increasing release of Gpr94-loaded extracellular vesicles from SMCs and matrix deposition of Gpr94 (Tsurumaki et al., 2020). GPR94 mRNA was overexpressed in pancreatic islets from patients with type 2 diabetes (Ghassay et al., 2019). |
| Eukaryotic translation initiation factor 3 subunit M (EIF3 M) | 5 | Translation initiation factor | Component of the eIF-3 complex required for initiation of protein biosynthesis | Endoplasm (ENPL, synonym: Grp94, gene name: Hsp90b1) |
| Endoplasm (ENPL, synonym: Grp94, gene name: Hsp90b1) | 5 | Molecular chaperone | Processing and transport of secreted proteins | None found |
| RNA-binding protein with serine-rich domain 1 (RNP51) | 5 | mRNA 3′-UTR binding | mRNA processing, mRNA splicing, Nonsense-mediated mRNA decay | RNP51 expression was up-regulated in the brains of ischemic stroke mice. Knockdown of RNP51 aggravated ischemic brain injury after middle cerebral artery occlusion (MCAO) and promoted neuronal death (Zhang et al., 2020a,b). |

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## Table 4 (continued)

| Protein name | Protein-Protein interactions (STRING) | Molecular function (UniProt) | Biological Process (UniProt) | Relevance to aging, hypertension or cerebrovascular function (PubMed or Present Study) |
|--------------|--------------------------------------|------------------------------|------------------------------|--------------------------------------------------------------------------------------|
| Microtubule-associated protein RP/EB family member 1 (MARE1, gene name Mapre1) | 4 | Microtubule plus-end binding | Cell cycle, Cell division, Mitosis | Proper gap junction trafficking requires Myocardin-Related Transcription Factor-dependent transcription of Mapre1 in ventricular cardiomyocytes (Trembley et al., 2019). |
| Exportin-1 (XPO1, synonym: Crm1) | 4 | RNA binding, protein binding | mRNA transport, protein export from nucleus | Exportin-1 (Crm1) is involved in the NFATc3 (nuclear factor of activated T-cells) nuclear export process in mouse cerebral arterial smooth muscle cells (Gomez et al., 2003). Cell death induced by Angiotensin II in human endothelial cells is dependent on Exportin-1 mediated nuclear export of E3 ubiquitin ligase RNF146 (Sheng et al., 2018). |
| Alpha-soluble NSF attachment protein (SNAAL; synonym α-SNAP; gene name Napa) | 4 | soluble NSF attachment protein activity, SNARE binding, syntaxin binding | ER-Golgi transport, intracellular protein transport | α-SNAP regulates dynamic, on-site assembly and calcium selectivity of Orail calcium-release channels (Li et al., 2016). α-SNAP expression was decreased in temporal lobe epilepsy (TLE) patients and in a pilocarpine-induced rat TLE model (Xi et al., 2015).Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −4.70; P = 0.0002) (Present study) | Nitrte-induced vascular relaxation is largely due to nitrte reduction by α-SNAP oxidase to NO (Pinder et al., 2009). |
| Aldehyde oxide 1/2 (AOX1/AOX2) | 2 | Oxidoreductase | Oxidase with broad substrate specificity | | |
| Neural cell adhesion molecule 1 (L1CAM) | 2 | Protein binding | Cell adhesion, differentiation, neurogenesis | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.21; P = 0.039) (Present study) | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.11; P = 0.049) (Present study) | FHL2 prevents cardiac hypertrophy in mice with cardiac-specific deletion of ROCK2 (Okamoto et al., 2013). FHL2 mediates podocyte Rac1 activation and foot process effacement in hypertensive nephropathy in mice (Li et al., 2019). Deficiency of FHL2 leads to delayed neuronal cell migration and premature astrocyte differentiation in mice (Kim et al., 2019). |
| Four and a half LIM domains protein 2 (FHL2) | 1 | Transcription factor activity and binding | Transcription regulation | | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.11; P = 0.049) (Present study) | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.11; P = 0.049) (Present study) | FHL2 prevents cardiac hypertrophy in mice with cardiac-specific deletion of ROCK2 (Okamoto et al., 2013). FHL2 mediates podocyte Rac1 activation and foot process effacement in hypertensive nephropathy in mice (Li et al., 2019). Deficiency of FHL2 leads to delayed neuronal cell migration and premature astrocyte differentiation in mice (Kim et al., 2019). |
| ADP-ribosylation factor 6 (Arf6) | 0 | GTP binding, GTPase activity | GTP-binding protein involved in protein trafficking | | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.75; P = 0.035) (Present study) | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.75; P = 0.035) (Present study) | ARF6 is involved in the organization of caveolae, ROS-dependent VEGF signaling, and angiogenesis (Issada et al., 2005). The GTPase ARF6 controls ROS production to mediate angiotension II-induced vascular smooth muscle cell proliferation (Bouroumoum et al., 2016). β-Arrestin-mediated angiotension II signaling controls the activation of ARF6 protein and endocytosis in migration of vascular smooth muscle cells (Charles et al., 2016). ARF6 inhibition stabilizes the vasculature and enhances survival during endotoxic shock (Davis et al., 2014). Arf6 in lymphatic endothelial cells regulates lymphangiogenesis by controlling directional cell migration (Lin et al., 2017).Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.26; P = 0.047) (Present study) | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.26; P = 0.047) (Present study) | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.26; P = 0.047) (Present study) |
| Extracellular matrix protein 2 (Ecm2) | 0 | Collagen binding | Extracellular matrix organization. Promotes matrix assembly and cell adhesiveness. | | | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.24; P = 0.026) (Present study) | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.24; P = 0.026) (Present study) | Inhibition of ROCK1 pathway is involved in the therapeutic effects of simvastatin on pulmonary arterial hypertension (Luo et al., 2013). ROCK1 is increased in Alzheimer’s disease and ROCK1 depletion reduces amyloid-β levels in brain (Henderson et al., 2016). Long-term beneficial effects of ROCK1 deficiency in hypertrophic decompensation in mice suggests ROCK1 as a therapeutic target to limit heart failure progression (Jin et al., 2010). ROCK1 is involved in small artery remodeling in obese, diabetic minipigs (Ludvigsen et al., 2019). Overactive ROCK1 in endothelial cell-cell junctions is specifically involved in and is a novel target for treatment of cerebral cavernous malformation disease (Gorszewska et al., 2016). Silencing of ROCK1 in aortic smooth muscle cells changes the cell morphology to a smaller cell area, fewer stress fibers, and increased number of focal adhesions (Wang et al., 2009). |
| Rho-associated protein kinase 1 (ROCK1) | 0 | Rho-dependent protein serine/threonine kinase activity | Regulator of actin cytoskeleton and cell polarity. Rho protein signal transduction | | | | Significantly down-regulated by aging in cerebral and mesenteric small arteries (Log2FC = −1.24; P = 0.026) (Present study) | Inhibition of ROCK1 pathway is involved in the therapeutic effects of simvastatin on pulmonary arterial hypertension (Luo et al., 2013). ROCK1 is increased in Alzheimer’s disease and ROCK1 depletion reduces amyloid-β levels in brain (Henderson et al., 2016). Long-term beneficial effects of ROCK1 deficiency in hypertrophic decompensation in mice suggests ROCK1 as a therapeutic target to limit heart failure progression (Jin et al., 2010). ROCK1 is involved in small artery remodeling in obese, diabetic minipigs (Ludvigsen et al., 2019). Overactive ROCK1 in endothelial cell-cell junctions is specifically involved in and is a novel target for treatment of cerebral cavernous malformation disease (Gorszewska et al., 2016). Silencing of ROCK1 in aortic smooth muscle cells changes the cell morphology to a smaller cell area, fewer stress fibers, and increased number of focal adhesions (Wang et al., 2009). |

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oxidase subunit 5B (COX5B) was downregulated in hypertrophied cardiac muscle in hypertensive rats (Kuo et al., 2005), leading to the suggestion that its vascular downregulation by age (Tables 1 and 3) might be involved in coronary microvascular rarefaction in heart failure and hypertrophy of resistance arteries in aged individuals.

In cluster 4 (Table 4) a total of 28 proteins are listed because they have several protein-protein interactions or their expression was significantly changed by age. Of these, 14 proteins (RS6, HSP7C, RSSA, ARPC2, EIF3 M, ENPL/Grp94, MARE1, XPO1/Crm1, SNAA/α-SNAP, AOX1/3, FHL2, Arf6, ROCK1, FBLN4) were judged as physiologically relevant for vascular structure and function based on the annotated information from literature searches (Table 4). The selected proteins are involved in transcription regulation (FHL2, HSP7C), translation and protein biosynthesis (RS6, RSSA, EIF3 M, ENPL/Grp94), intracellular protein processing/transport/trafficking (Arf6, MARE1, XPO1/Crm1, SNAA/α-SNAP), metabolic pathways (AOX1/3, AOX2), actin cytoskeleton regulation (ARPC2, ROCK1), and extracellular elastic fiber assembly (FBLN4). Of these, AOX1/3 are directly involved in NO-mediated vasodilator responses (Pinder et al., 2009), and several proteins (EIF3 M, FHL2, ROCK1, FBLN4) play a role in vascular structure and/or remodeling of the extracellular matrix or cytoskeleton (Li et al., 2019; Ludwigsen et al., 2019; Okamoto et al., 2013; Pang et al., 2020; Wang et al., 2009). In support of the latter findings, age-associated proteomic changes in human aortic media (smooth muscle layer) were characterized by upregulation of 5 markers of oxidative stress and differential regulation of 14 extracellular matrix proteins, suggesting a role in
structural remodeling of the aging aortic wall (Miura et al., 2019). This changed or clustered by age. Highlighted in bold are highly significantly (Ludvigsen et al., 2019), endotoxic shock (Arf6) (Davis et al., 2014), neointima formation (ROCK1) (Noma et al., 2008), diabetes (ROCK1) late structural vascular remodeling (Corada et al., 2010). Recent development and angiogenesis (Foulquier et al., 2018) and can modulate for the calcium sensitization process involved in vascular smooth muscle cells. The latter could indicate a tendency to a hypercontractile state of vascular smooth muscle cells in middle-aged mice. However, it might also function as a compensation for the loss of ROCK1 expression by counteracting the reduced inhibition of MLCP by ROCK1. Furthermore, posttranslational modifications affecting ROCK1 activity should be considered in future studies. Interestingly, the most up-regulated protein by age was Wnt-10a. As shown above ROCK1 is implicated in a number of processes in vascular structure and diseases, whereas the ROCK2 isoform seems to be responsible for the calcium sensitization process involved in vascular smooth muscle cell contractility (Wang et al., 2009). M-RIP/p116<sup>RB</sup> is a scaffolding protein targeting RhoA to interact with MLCP, and therefore important for the contractile function in vascular smooth muscle cells (Koga and Ikebe, 2005; Surks et al., 2005). ROCK2 protein was not changed significantly by age in the present study, which is in contrast to our previous study showing increased mRNA expression of ROCK2 in mesenteric resistance arteries from middle-aged mice (Börjling et al., 2018). However, ROCK2 activity might still be upregulated in aging, for example via posttranslational modifications. The functional consequences of down-regulation of M-RIP and ROCK1 by aging in the present study deserve attention. While reduced ROCK1 expression could indicate a lowered risk of remodeling and fibrosis in middle-aged arteries, a lack of M-RIP would limit dephosphorylation of the regulatory light chain (MLC<sub>90</sub>) of myosin II, increase actin polymerization, and increase the number of stress fibers in vascular smooth muscle cells. The latter could indicate a tendency to a hypercontractile state of vascular smooth muscle cells in middle-aged mice. However, it might also function as a compensation for the loss of ROCK1 expression by counteracting the reduced inhibition of MLCP by ROCK1. Furthermore, posttranslational modifications affecting ROCK1 activity should be considered in future studies. Interestingly, the most up-regulated protein by age was Wnt-10a (Table 1) belonging to the canonical Wnt/beta-catenin pathway, which is activated during advanced arterial aging in humans (Marchand et al., 2011). The canonical Wnt/beta-catenin pathway controls vascular development and angiogenesis (Foulquier et al., 2018) and can modulate structural vascular remodeling (Corada et al., 2010). Recent evidence suggests a specific role for this pathway in age-related macular degeneration (Vallee et al., 2020).

The Vitamin B6 (pyridoxine) metabolism was the only enriched KEGG pathway based on the proteins significantly affected by age in the present study. This was due to a very potent down-regulation of the aldehyde oxidase isoforms 1 and 2 (see Table 1 and Fig. 4). Vitamin B6 deficiency was previously linked with increased homocysteine levels (hyperhomocysteinemia), aging and cardiovascular disease in humans (Wilcken and Wilcken, 1998), but recent case-controlled studies have dismissed this hypothesis (Friso et al., 2012). In rats, however, vitamin B6 deficiency leads to hypertension and was used to create a hypertensive rat strain (Dakshinamurti et al., 1998). This suggests that there might be a hypertensive effect in rodents of the age-dependent down-regulation of vitamin B6 metabolism. Mild hyperhomocysteinemia is common in older people and has been identified as a risk factor in development of Alzheimer’s disease (Hainsworth et al., 2016; Seshadri et al., 2002; Sudduth et al., 2013). Vitamin B6 is a cofactor in conversion of homocysteine to cystathionine and cysteine, and, together with Vitamin B12, it is an important dietary supplement to prevent hyperhomocysteinemia in the elderly (Hainsworth et al., 2016). On the other hand, a metaanalysis with cognitive data on 22,000 individuals concluded that lowering of plasma homocysteine by using B vitamins for ~5 years had no significant effect on individual cognitive domains, global cognitive function, or cognitive aging (Clarke et al., 2014). How exactly Vitamin B6 deficiency affects vascular structure and function, especially in the brain, is nevertheless a subject worthy of future investigations.

Using a list of significantly changed and clustered proteins due to age we obtained a number of enriched KEGG pathways as shown in Table 5. "Biosynthesis of antibiotics" pathway might be relevant for changes in the innate and adaptive immune responses in aged individuals, which is interesting in the context of SARS-CoV-2 infection and the strong correlation between Covid-19 severity and age. The enrichment of this pathway is largely confirmed in a single-cell transcriptomic study of mouse brain endothelial cells, finding that innate immunity and antigen processing pathways were upregulated in aged mouse brain capillaries (Chen et al., 2020). Since changes in the immune system and inflammatory pathways are involved in hypertension (Rodríguez-Iiturbe et al., 2017), future studies should address the relevance of the "Biosynthesis of antibiotics pathway for aging of small arteries. Regulation of actin cytoskeleton and Endocytosis pathways deserve attention in relation to vascular structure and function. In total 12 proteins from our input list were found enriched with the Regulation of actin cytoskeleton pathway. As depicted in Fig. 5A, these proteins have widespread functions from activation of membrane-bound receptors, cell migration, adherens junctions, RhoA/Rho-kinase signaling, actin polymerization and stress fiber formation. The combined effects of the age-dependent changes are difficult to assess and further studies need to clarify the impact of these changes through pharmacological or genetic targeting of the individual proteins. However, as previously discussed the down-regulation of M-RIP/p116<sup>RB</sup> would be an interesting and novel target for investigating age-dependent changes in resistance artery function and possibly hypertension. In Fig. 5B is shown the Endocytosis pathway containing 11 proteins with an age-dependency in our bioinformatics analyses. These 11 proteins function in the initiation of clathrin-dependent and -independent endocytosis and in stabilization of caveolae, while other proteins are involved in early and late endosomal pathways or in vesicle recycling (Fig. 5B). The GTP-binding protein Arf6, which is involved in intracellular protein trafficking, is relevant for vascular structure and function as it plays diverse roles in VEGF signaling, angiogenesis/lymphangiogenesis, ROS production, and VSMC migration (see annotations in Table 4). Furthermore, caveolin-2 may play a role in aging by controlling caveolae. Studies indicate that the ability of Cav3.2 T-type calcium channels to initiate BK<sub>Ca</sub> channel dependent negative feedback vasodilatation relies on intact caveolae (Harraz et al., 2014; Harraz and Jensen, 2020). This negative feedback mechanism was shown to be

### Table 5

| Enriched KEGG Pathway | % Occurrence in data set | P-value |
|-----------------------|-------------------------|---------|
| Biosynthesis of antibiotics | 6.3 | 0.000005 |
| Regulation of actin cytoskeleton | 5.8 | 0.00035 |
| Carbon metabolism | 3.9 | 0.0018 |
| Biosynthesis of amino acids | 2.9 | 0.0056 |
| Endocytosis | 5.3 | 0.0059 |
| Bacterial invasion of epithelial cells | 2.9 | 0.0063 |
| Valine, leucine and isoleucine degradation | 2.4 | 0.0093 |
| Huntington’s disease | 4.4 | 0.0099 |
| Proteoglycans in cancer | 4.4 | 0.011 |
| Focal adhesion | 4.4 | 0.013 |
| Glycogenolysis / Gluconeogenesis | 2.4 | 0.017 |
| Ribosome | 3.4 | 0.022 |
| Parkinson’s disease | 3.4 | 0.025 |
| Non-alcoholic fatty liver disease (NAFLD) | 3.4 | 0.031 |
| Protein processing in endoplasmic reticulum | 3.4 | 0.041 |
| RNA transport | 3.4 | 0.043 |
| ECM-receptor interaction | 2.4 | 0.044 |
Fig. 5. A-B: KEGG pathway analysis of proteins clustered in an age-dependent manner (Cluster 4 + subcluster 2) including the set of proteins significantly affected by age ($P < 0.05$) (in total 207 proteins). In this analysis 17 pathways were significantly enriched ($P < 0.05$; See Table 5). Of these, 3 pathways had an occurrence of proteins >5% and at the same time were highly significantly enriched ($P < 0.01$). These pathways are highlighted with red color in Table 5. For simplicity, we show here the two pathways with relevance for vascular structure and vasomotor responses: Regulation of actin cytoskeleton (A) and Endocytosis (B). The red dots in the two figures indicate where proteins affected by age are positioned in the respective pathways. See also Tables 5 and 6.
lacking in mesenteric resistance arteries from young mice deficient in Ca$_3$T-type channels, as well as in middle-aged wild-type mice (Mikkelsen et al., 2016). Finally, a recent study showed that aged mice have a smaller density of caveolae, which could explain the lack of contractility in middle cerebral arteries compared to the mesenteric resistance arteries (Fan et al., 2020; Harraz and Jensen, 2020). In caveolin-2 deficient mice, rapid onset dilatation in skeletal muscle arterioles was reduced by 50% with a concomitant 40% increase in skeletal muscle contractile force (Fernando et al., 2016). Whether and how this result is related to aging-induced loss of muscle strength would require further studies. On the other hand, in cerebral artery myocytes from rats the caveolin-1 and -3 were the abundant isoforms involved in regulation of intracellular calcium homeostasis (Kamishima et al., 2007). It should be noted that our mass spectrometry study does not detect posttranslational modifications of proteins, so we would not be able to detect altered glycation endproducts (Simm, 2013) or altered phosphorylation status (Ferrer et al., 2021; Gannon et al., 2008) of proteins affected by age in the small arteries.

We found 16 enriched KEGG pathways based on proteins differentially expressed in middle cerebral arteries vs. mesenteric resistance arteries (Table 6). Of these, 7 pathways had a protein occurrence over 5% and were highly significant (P < 0.01). Protein digestion and absorption, Oxidative phosphorylation, and Focal adhesion were the top 3 enriched pathways, whereas various disease pathways formed the remaining four. It is interesting that Focal adhesion pathway is differentially expressed in the two vascular beds as it is also part of the Regulation of actin cytoskeleton pathway, which was enriched in the age-dependent analyses. If we focus on the list of differentially expressed proteins in the two vascular beds (Table 2), we do not find that any of the proteins were significantly affected by age. However, it is interesting to note that the α$_2$-subunit of the Na/K-ATPase is much higher expressed in middle cerebral arteries compared to the mesenteric resistance arteries, whereas the α$_1$-subunit is much lower expressed. The α$_2$-subunit is more sensitive to ouabain than the α$_1$-subunit (being rather ouabain-insensitive), and the differential expression of the two isoforms in middle cerebral arteries and mesenteric resistance arteries could have implications for studies of the role of the Na/K-ATPase subunits in hypertension (Dostanic-Larson et al., 2006; Oshiro et al., 2010).

5. Conclusion

In conclusion, more proteins were significantly affected by vessel type than by aging in middle cerebral arteries and mesenteric resistance arteries in mice. However, the proteomic changes due to age were shown to comprise a number of potentially important proteins for vascular structure and function. The proteomic data clustered in an age-dependent manner, and we could identify a number of protein-protein interaction networks with potential importance for age-dependent changes in the resistance vasculature. Our KEGG pathway analyses revealed that Vitamin B6 metabolism, Biosynthesis of antibiotics, Regulation of actin cytoskeleton, and Endocytosis were the top enriched pathways due to age. A number of important age-dependent changes occurred in proteins involved in extracellular matrix organization and intracellular cytoskeleton structure and regulation, with the RhoA/Rho-kinase pathway being at the center of these changes. We conclude that ROCK1 and M-RIP/p116<sup>pro</sup> along with other members of the Regulation of actin cytoskeleton pathway are important targets for future studies on age-dependent hypertension and dysregulation of blood flow in critical organs such as the brain.

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