The Effects of Resveratrol on Endothelial Function of Ageing Arteries

By

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Abstract

Ageing is a major risk factor for the development of endothelial dysfunction and cardiovascular disease (CVD). In 2014, CVD was the second most common cause of death in the UK. Clearly, the socio-economic burden of CVD is significant and there is a considerable need for lifestyle modifications, such as nutraceutical supplementation that help ameliorate its severity. Here, we investigated the effects of resveratrol (RV), a polyphenol present in red grapes, berries and peanuts on endothelial function during ageing. There is a murine (Chapters 2-3) and a human (Chapter 4) component to these studies. The aim of the murine component was to establish the vasodilator response during ageing (4 – 26 months of age) of the isolated pressurized mouse femoral artery to pharmacological and mechanical stimuli following incubation with RV. The human component aimed to determine the effects of supplementation with RV on endothelial function and oxygen consumption (VO_2) in coronary artery disease (CAD) elderly patients. Hence, the animal study aimed to provide mechanistic insight into the vascular and physiological parameters assessed in humans. In Chapter 2, we describe that the isolated pressurized femoral artery of 26-month old mice does not develop compromised endothelial function when compared to that of 4-month old mice, as reflected by similar levels of dilation in response to acetylcholine (ACh; 10^{-9} – 10^{-3} M) and intraluminal flow (5-10 $\mu L \cdot min^{-1}$). Despite similar levels of flow-mediated dilation (FMD) of the isolated pressurized femoral artery of young and old mice, there are differences in the role that endothelial factors play in dilation with ageing; addition of L-NG-nitro-L-arginine (LNNA; 100 μM)

or apamin (100 nM) + Tram 34 (1 uM) significantly reduced dilation regardless of age. However, addition of indomethacin (10 uM) had no effect on FMD in the young whereas it abolished it in the old. The isolated pressurized mouse femoral artery, regardless of age, shows an initial maximal increase in diameter followed by a sustained plateau phase in response to intraluminal flow. In the presence of flow, the artery auto-regulates its diameter back to pre-constricted levels. Such capacity for auto-regulation is reduced upon addition of LNNA, apamin + Tram 34 or indomethacin. In Chapter 3, we report that RV causes differential effects on ACh-induced and FMD of the isolated pressurized mouse femoral artery; whereas it increases ACh-induced dilation it compromises FMD. RV on its own and in combination with inhibitors of endothelial factors reduce the capacity of the isolated pressurized mouse femoral artery to auto-regulate diameter in the presence of intraluminal flow. The effects of RV on FMD indicate that RV might compromise flow-mediated endothelial mechanotransduction. In Chapter 4, we describe how acute supplementation with high doses of RV (~1 g·day for three days) failed to improve FMD of the brachial artery or VO_2 kinetics in elderly CAD patients. Our findings suggest important age-related effects on the mechanisms regulating arterial tone despite preserved degrees of relaxation. Secondly, they raise important questions regarding the insight gained from *ex-vivo* models. Investigating vasodilation by means of agonists (i.e., ACh) might not provide a comprehensive understanding of the effects of RV on endothelium-dependent dilation. Finally, they question previous *in-vitro* studies and suggest no significant benefits from acute and high doses of RV on cardiovascular function of older CAD patients.

Acknowledgments

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Ethical Consideration for Experimental Animals

The experiments with pressure myography presented in this thesis were performed using animal tissue excised from dead animals (C57BL/6 male mice, 28 – 30 g in weight, aged 4 and 26 months), obtained from Charles Rivers (UK) and maintained at the BSU, University of Manchester. The mice were housed under standardised conditions (12 hour light/dark cycles at 24°C). The animals were humanely killed by cervical dislocation. All procedures were conducted in accordance with institutional guidelines of MMU and the University of Manchester and the United Kingdom Animals (Scientific Procedures) Act of 1986 (schedule 1). A risk assessment for the use and handling of animal tissue was conducted.

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List of Abbreviations

ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
Ang-II	Angiotensin II
ANOVA	Analysis of variance
BH_4	Tetrahydrobiopterin
BP	Blood pressure
Ca^{2+}	Calcium
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
cGMP	Guanosine 3',5 -cyclic monophosphate
CNP	C-type natriuretic peptide
COXs	Cyclooxygenases
CVD	Cardiovascular disease
CYP450	Cytochrome P450
DMSO	Dimethyl sulfoxide
EC	Endothelial cells
ED	Endothelial dysfunction
EDHFs	Endothelial-derived hyperpolarizing factors
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1

FAD	Flavin adenine dinucleotide
FMD	Flow-mediated dilation
FMN	Flavin mononucleotide
H_2O_2	Hydrogen peroxide
HCAEC	Human coronary artery endothelial cells
HPLC	High-performance liquid chromatography
IGF-1	Insulin-like growth factor 1
iNOS	Inducible nitric oxide synthase
IP3	Inositol 1,4,5-trisphosphate
K^+	Potassium ions
K_{Ca} channels	Calcium-activated potassium channels
KPSS	High potassium physiological salt solution
LNNA	L-NG-nitro-L-arginine
MRT	Mean response time
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\cdot -}$	Anion superoxide
$ONOO^-$	Peroxynitrite
PBS	Phosphate buffered saline
PECAM-1	Platelet endothelial cell adhesion molecule-1

PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGG2	Prostaglandin G2
PGI_2	Prostacyclin
Phe	Phenylephrine
PKG	Protein kinase G
PKG-1	Protein kinase G-1
PSS	Physiological salt solution
PTGIS	Prostacyclin synthase
ROS	Reactive oxygen species
RV	Resveratrol
SEM	Standard error of mean
SHR	Spontaneously hypertensive rats
TXA_2	Thromboxane 2
TNF-α	Tumour necrosis factor
VO_2	Oxygen consumption
VSMCs	Vascular smooth muscle cells

Foreword

Move Age; An Erasmus Mundus doctorate programme

Move Age is a joint doctorate program funded by European Commissions as part of the umbrella program Erasmus Mundus. Through original research, Move Age aims to provide a scientific and comprehensive framework for the understanding of ageing and to develop strategies that translate into a better quality of life for the elderly. The research projects that comprise the program take place in at least two of the three participating institutions in Europe; Manchester Metropolitan University (MMU; England), the Catholic University of Leuven (KU Leuven; Belgium) and VU University (The Netherlands). The findings presented in this thesis are the result of the research I conducted in MMU and KU Leuven from 2013 to 2016 and involve data from murine and human studies. In these studies, I aimed to characterize the dilator response of the isolated pressurized mouse femoral artery and determine the potential of resveratrol (an antioxidant) to improve vessel dilation in both mice and humans. The following sections explain from a biological point of view the mechanisms that drive the ageing process in the vasculature, the burden of ageing for public health and the rationale behind the use of nutraceutical strategies to improve cardiovascular health in the elderly.

Introduction

Vascular anatomy and physiology

Nutrients, oxygenated blood, and waste flow to, and from the organs through the blood vessels. Blood vessels can be broadly categorized into three types. First, arteries, which are wide and carry oxygenated blood to the tissues from the heart. Secondly, capillaries, which are narrow and allow for the actual exchange of nutrients into and from the tissues. Lastly, veins, which are also wide and superficial and carry deoxygenated blood and waste back to the heart. Exemptions to this nomenclature exist and include the pulmonary and the umbilical arteries, which carry deoxygenated blood from the heart to the lungs and from the foetus to its mother, respectively. Three histologically different layers compose the blood vessels. Each region contains particular amounts of vascular smooth muscle cells (VSMCs) and elastin (Ruoslahti and Engvall, 1997; Pugsley and Tabrizchi, 2000). The tunica externa is the outermost layer of the blood vessel and contains mainly collagen, which stabilizes the vessels near an organ. The tunica media is the middle layer of the vessel and is composed of smooth muscle and elastic tissue. The tunica media in arteries contain more smooth muscle tissue than in veins since most arteries regulate blood flow by means of smooth muscle contraction. The innermost layer of the blood vessel is the tunica intima and consists mainly of one single layer of endothelial cells (EC) in direct contact with the blood. Arteries are further classified into conduit and resistance

arteries depending on the amount of smooth muscle or collagen in their tunica media. Large arteries such as the aorta are considered conduit arteries and present a larger amount of collagen, which allows them to stretch with each pulse (Pugsley and Tabrizchi, 2000). Figure 1 shows the anatomy of the blood vessel.

Blood flow through the circulatory system is a relatively passive process, which is largely determined by the pumping action of the heart. However, as mentioned above, blood vessels and in particular resistance arteries, help regulate blood flow via the contraction and relaxation of the smooth muscle in their tunica media. Regulation of the radii in blood vessels allows for the difference in pressure necessary for blood flow to occur. Many factors regulate the dilation and constriction of the blood vessels. Extrinsic factors include noise, light and temperature whereas intrinsic factors comprise sympathetic and hormonal control, oxygen saturation and skeletal muscle work (Dinenno et al., 2000; Kräuchi et al., 2000; Kurcer et al., 2006; Sato and Ooishi, 2012). The vascular endothelium is critical in modulating vessel diameter by means of secretion of vasodilating and vasoconstricting molecules. For the purpose of this thesis, we will focus here on the vasodilating substances released by the vascular endothelium.

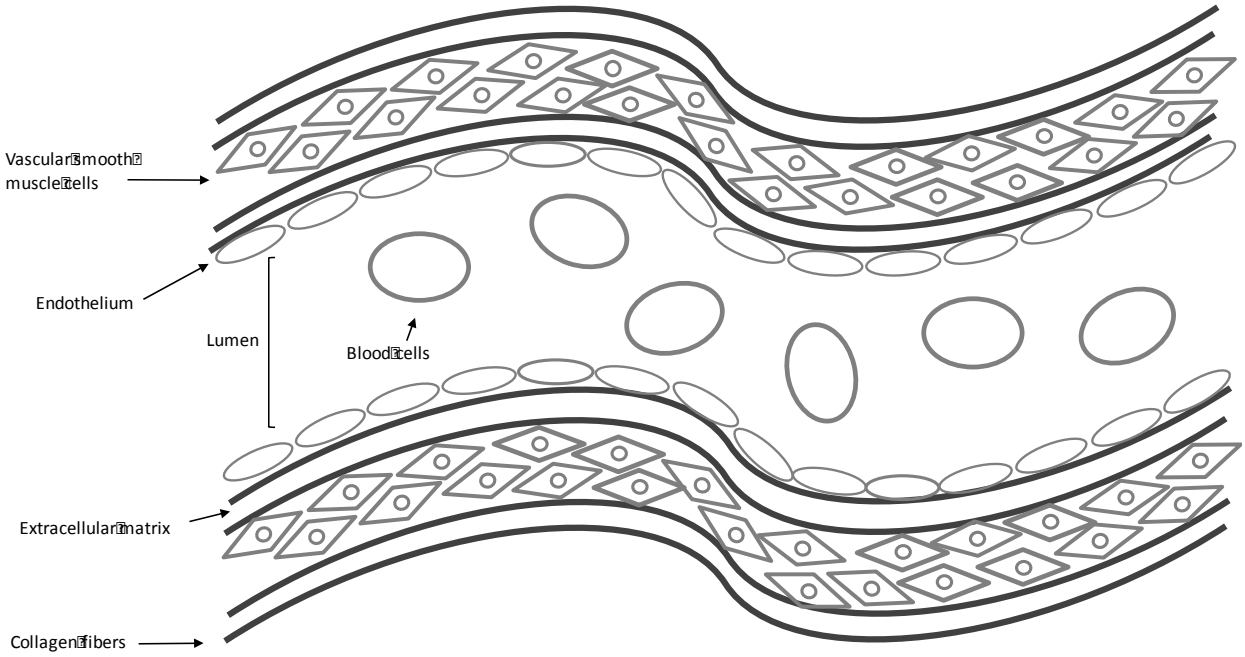

FIGURE 1. SCHEMATIC REPRESENTATION OF THE ANATOMICAL ORGANIZATION OF THE BLOOD VESSEL (author generated).

The vascular endothelium

The vascular endothelium is a monolayer of cells that lines the inner wall of the vessel. It is a secretory tissue that responds to a broad variety of chemical and mechanical stimuli and as such, plays a pivotal role in both constriction and dilation of the blood vessel. Although not discussed here, the endothelium is also critical in processes such as coagulation and fibrinolysis (Risau, 1995). The balance between constriction and dilation allows for the appropriate delivery of nutrients and oxygen as well as the removal of waste to, and from the organs. EC lie adjacent to VSMCs, and they communicate directly with each other. Not only does the interaction between EC and VSMCs allow for vessel dilation and constriction, it also regulates VSMCs proliferation and EC migration (Campbell and Campbell, 1986). Under basal conditions, resistance arteries and arterioles exhibit some degree of constriction known as vascular or myogenic tone. Such tone creates the resistance necessary for blood to flow through the system (Shimoda et al., 2000). Vascular tone exists in the absence of a functional endothelium as it has been demonstrated in *ex-vivo* models (Azzawi and Austin, 2006). However, EC help regulate vascular tone through the release of vasoactive substances; vasodilators and vasoconstrictors (Vincent et al., 2000).

Endothelium-dependent vasodilation

Vessel dilation is critical for cardiovascular health. Loss of vessel dilation, for instance, correlates with the onset of atherosclerosis and the development of cardiovascular disease (CVD). Endothelium-dependent vasodilation is an intricate and highly orchestrated process. It is the result of the interaction of at least three (types of) molecules: nitric oxide (NO), prostacyclin (PGI_2), and endothelial-derived hyperpolarizing factors (EDHFs), such as hydrogen peroxide (H_2O_2) (Triulzi et al., 1981; Busse et al., 1984; Furchgott and Vanhoutte, 1989; Bellien et al., 2008).

Nitric oxide

NO is an extremely short-lived (~1s), naturally occurring gaseous molecule synthetized from L-arginine, oxygen, nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4), haem and calmodulin by NO synthase (NOS) (Pacher et al., 2007; Lundberg et al., 2008). In mammals, there are three isoforms of NOS. Neuronal (nNOS), inducible (iNOS), and endothelial NOS (eNOS). nNOS is found predominantly in the in nervous tissue and type 2 skeletal muscle fibres. eNOS, on the other hand, is mostly expressed in EC, the heart, brain and kidney. Lastly, iNOS is not constitutively

expressed in normal quiescent cells. Instead, iNOS is expressed in virtually all cell types upon exposure of the cells to bacteria or inflammatory cytokines (Förstermann and Sessa, 2012). Whereas nNOS and iNOS are mainly soluble and found in the cytoplasm (some exceptions to this exist; brain nNOS for instance is found in particulate form too), eNOS is membrane-bound. NO plays important signalling roles in a broad range of physiological processes. Although mostly known for its vasodilating properties, NO also prevents platelet aggregation and leucocyte adhesion to the endothelium (Cooper, 1999). Immune cells secrete NO as a defence mechanism against some bacteria and pathogens since NO can cause DNA damage and destruction of iron-sulfur centres (Niedbala et al., 2006). As a vasodilator, NO activates guanylyl cyclase in the VSMCs increasing the concentration of guanosine 3',5 -cyclic monophosphate (cGMP). cGMP in turn, activates protein kinase G (PKG), which leads to the opening of calcium-activated potassium (K_{Ca}) channels and the consequent uptake of calcium (Ca^{2+}). Ca^{2+} is necessary for the contractile activity of actin and myosin. Hence, the decrease in the levels of intracellular Ca^{2+} promotes smooth muscle cell relaxation and eventually, vessel dilation. In mammals, NO can also be synthesized from nitrite via the nitrate-nitrite-NO pathway. The half-life of circulating nitrate is considerably longer than NO. Hence, circulating nitrate serves as a pool of NO. Nitrate can be converted into nitrite under acidic and hypoxic conditions such as digestion in the gastrointestinal tract or muscle contraction during exercise, respectively. Nitrite can be converted to NO by haemoproteins such as haemoglobin, and myoglobin (Shiva, 2013). A basal production of NO is necessary to maintain tone and the adequate functioning of the blood vessel. Increases in shear stress or

sympathetic stimulus lead to higher production of NO and hence, vessel dilation (Dawes et al., 1997). Figure 2 exemplifies the production of NO by NOS.

Prostacyclin

Similar to NO, PGI_2 inhibits platelet activation and is an important vasodilator. PGI_2 belongs to a family of lipid molecules known as eicosanoids. Eicosanoids are produced by the hydrolysis of cellular membrane phospholipids by phospholipase A_2 and the consequent action of cyclooxygenases (COXs) and PGI_2 synthase (PTGIS) (Majed and Khalil, 2012). To date, three isoforms of COXs have been identified. All of the three isoforms convert arachidonic acid to prostaglandins. However, whereas COX-1 is a constitutive enzyme and is responsible for the production of baseline levels of prostaglandins, COX-2 is a cytokine-inducible form and produces prostaglandins that mediate inflammation. The exact function of COX-3 in humans has not yet been elucidated. COX-1 produces prostaglandin G2 (PGG2) from free arachidonic acid. PGG2 is a metabolic intermediate and the substrate of PTGIS for the production of PGI_2 in the EC (Majed and Khalil, 2012). Figure 3 shows the metabolic pathway for the synthesis of PGI_2. The mechanisms by which PGI_2 promotes vasodilation are similar to NO. PGI_2 acts downstream a paracrine signalling cascade that results in the relaxation of smooth muscle. It is released by EC in response to hypoxia or shear

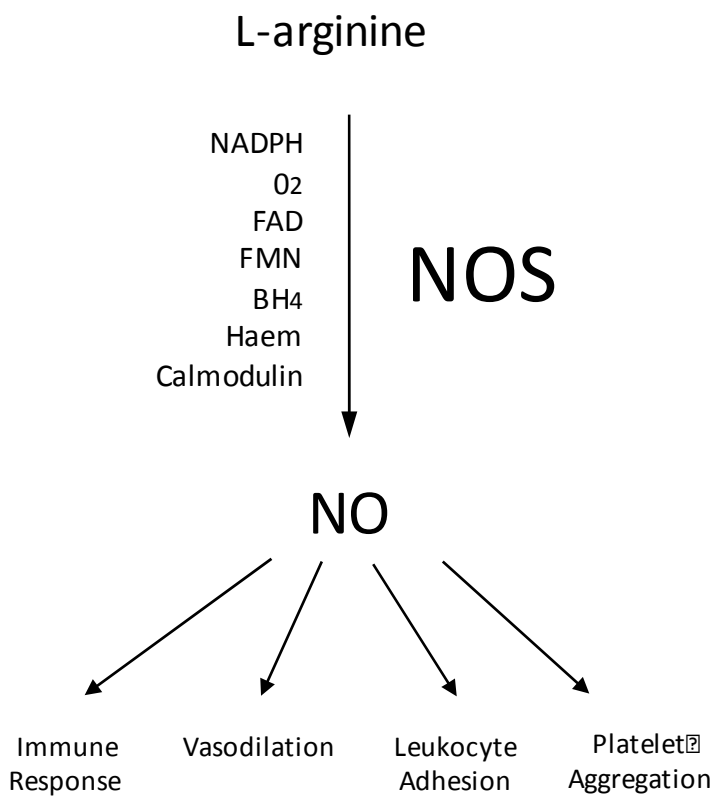

FIGURE 2 SCHEMATIC REPRESENTATION OF THE ENZYMATIC PRODUCTION AND FUNCTIONS OF NO IN THE VASCULATURE.

Nicotinamide adenine dinucleotide phosphate (NADPH); Oxygen (O_2); Flavin adenine dinucleotide (FAD); Flavin mononucleotide (FMN); Tetrahydrobiopterin (BH_4); Nitric oxide (NO), Nitric oxide synthase (NOS). Adapted from (Lundberg et al., 2008).

stress (Hanada et al., 2000). When bound to its receptor in the VSMCs, PGI_2 activates adenylyl cyclase and increases the levels of cAMP. As a second messenger, cAMP activates protein kinase A (PKA), which leads to the uptake of Ca^{2+}, inhibition of myosin ATPase and eventually, blood vessel relaxation (Majed and Khalil, 2012).

Endothelium-derived hyperpolarizing factors

In some vascular beds, when the production of NO and PGI_2 is pharmacologically inhibited, a certain degree of vasodilation is still present in response to mechanical or chemical stimuli (Csányi et al., 2012; Kang, 2014; Matsumoto et al., 2016). This is due to the contribution of EDHFs. As hyperpolarizing agents, EDHFs act by decreasing the intracellular levels of potassium ions (K^+). Hence, by definition EDHFs can be either molecules or electrical signals originated in the endothelium that hyperpolarize and consequently, relax VSMCs (Luksha et al., 2009). The exact chemical nature of EDHFs is yet to be characterized. However, it is known that in addition to NO and PGI_2, at least other four molecules secreted by the EC bear vasodilating potential. These are: 1) products of cytochrome P450 (CYP450) (Yang et al., 2015), 2) K^+ (Hangaard et al., 2015), 3) C-type natriuretic peptide (CNP) (Villar et al., 2007) and 4) H_2O_2 (Shimokawa and Morikawa, 2005).

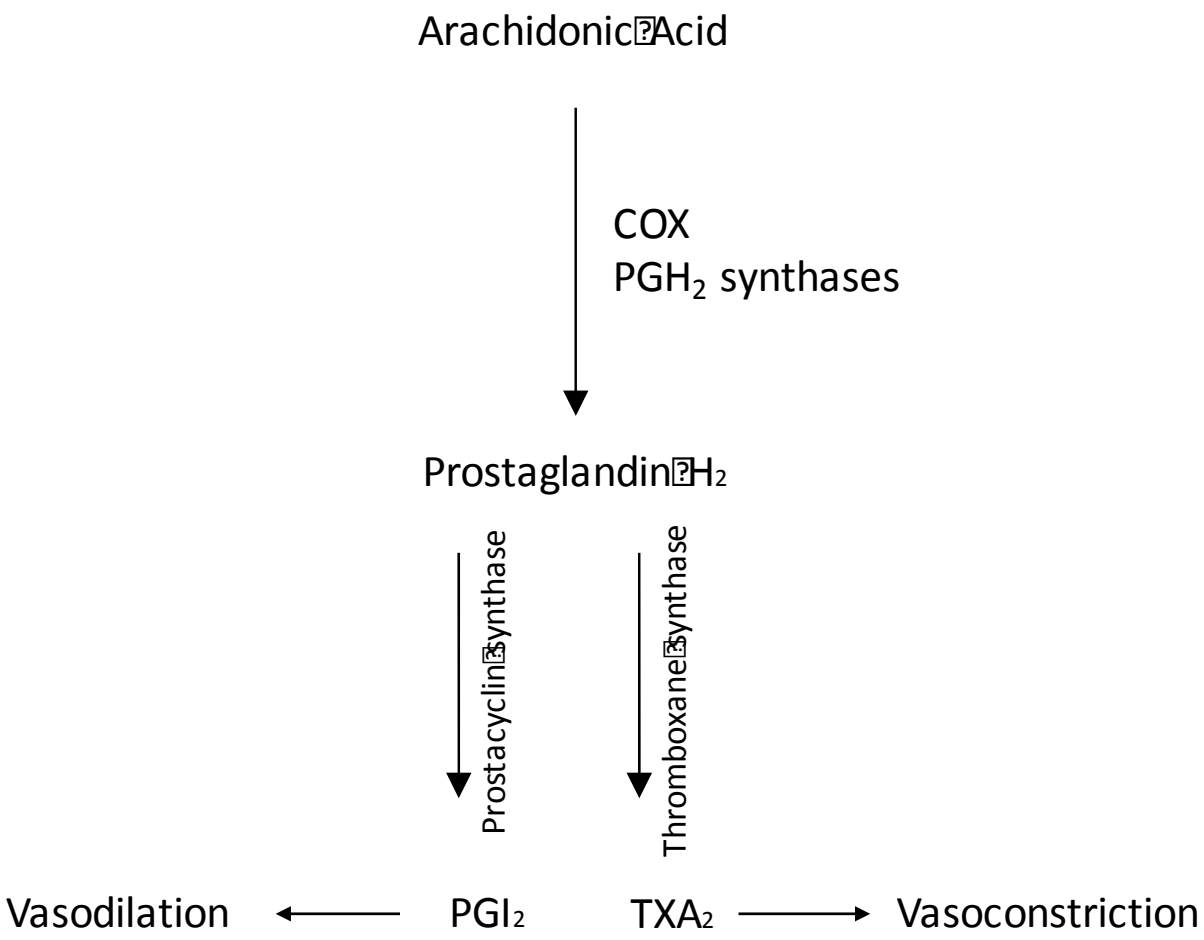

FIGURE 3. ENZYMATIC PATHWAY FOR THE SYNTHESIS AND FUNCTIONS OF PROSTACYCLIN (PGI_2) AND THROMBOXANE 2 (TXA_2).

Cyclooxygenase (COX); Prostaglandin H_2 (PGH_2). Adapted from (Brock and Peters-Golden, 2007).

Inhibition studies have revealed the role of ion channels in EDHF-mediated vasodilation. Similar to NO and PGI_2, decreases in the intracellular concentrations of Ca^{2+} are pivotal to the EDHF-mediated relaxation of smooth muscle. However, the mechanisms by which EC achieve such variation in the levels of Ca^{2+} are markedly different and do not necessarily involve cyclic nucleotides. EDHFs provoke the hyperpolarization of smooth muscle by activating both Ca^{2+} and K^+ -sensitive channels (Garland and Dora, 2017). Arachidonic acid, for instance, is a substrate of CYP450. Epoxyeicosatrienoic acids, products of the metabolism of arachidonic acid by CYP450, can directly activate KCa^{2+} channels leading to relaxation of smooth muscle. K^+ on its own can also activate KCa^{2+} channels, cause the efflux of K^+ into the myoendothelial gap junction, increase the intracellular levels of Ca^{2+} and hence, relax smooth muscle. Thirdly, CNP-mediated relaxation involves the activation of inwardly rectifying K^+ channels via the binding to the natriuretic peptide receptor. Finally, in the EC, the anion superoxide ($O_2{\cdot}^-$) is produced as a by-product by the mitochondria in response to increases in the levels of Ca^{2+}. H_2O_2 in turn, is the product of the dismutation of the O_2-. H_2O_2 too can directly activate KCa^{2+} channels causing smooth muscle relaxation (Garland and Dora, 2017).

Endothelium-dependent vasodilation is the result of the interplay of these molecules (and most likely others yet to be characterized). Depending on the vascular bed, the secretion of PGI_2 for instance, leads to further secretion of NO and vice-versa (Shimokawa et al., 1988). It is believed that EDHFs play a compensatory role in

dilation when the production of NO is compromised (Taddei et al., 1999). The secretion of these molecules in humans and other mammals is determined by the nature of the stimulus and the vascular bed. Not all vessels rely proportionally on the same molecules for dilation. For instance, it is well established that conduit vessels such as the aorta rely on NO more than EDHFs in mediating vascular dilation in contrast to coronary arteries that mainly depend on the latter, or a combination of both NO and EDHFs (Bauersachs et al., 1996). On the other hand, resistance arteries rely more on EDHFs. Hence, EDHFs are pivotal in the regulation of vascular resistance and blood pressure (BP) (Luksha et al., 2009). Moreover, the fact that at least three (types of) molecules can promote vasodilation sets in place redundant mechanisms that guarantee the appropriate functioning of the blood vessel in case the bioavailability of NO, or the secretion of PGI_2 and EDHFs are compromised. Figure 4 shows the interplay between the vasodilating agents released by the endothelium and how they promote vessel dilation.

The experimental studies that comprise this thesis deal with the potential of resveratrol (RV) to improve vasodilation. However, for the sake of context, vasoconstriction will be briefly discussed below.

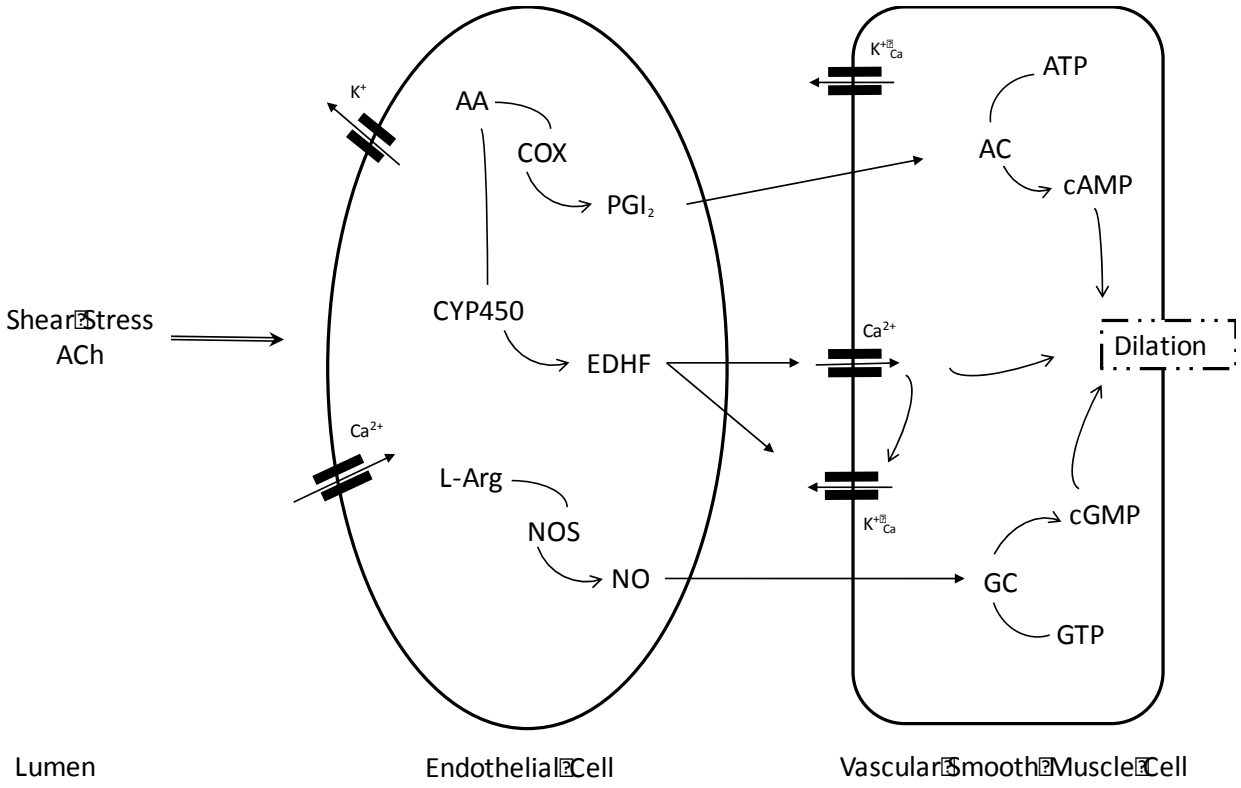

FIGURE 4. ENDOTHELIUM-DEPENDENT VASODILATION.

Mechanical or chemical stimuli such as increases in shear stress or acetylcholine (ACh) from nerve terminals stimulate the production of nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factors (EDHFs). These molecules promote the relaxation of smooth muscle by means of second messengers and/or membrane hyperpolarization-dependent mechanisms, respectively. Arachidonic acid (AA); Cyclooxygenase (COX); Cytochrome P450 (CYP450); Nitric oxide synthase (NOS); L-Arginine (L-Arg); Adenylyl cyclase (AC); Guanylyl cyclase (GC); Large conductance calcium-activated potassium channels (K^+Ca); Cyclic adenosine monophosphate (cAMP); Cyclic guanosine monophosphate (cGMP). Adapted from: (Szchuman-Sapir 2014).

Vasoconstriction

Vasoconstriction is the narrowing of the blood vessels as a consequence of smooth muscle contraction. It results in higher vascular resistance and contrary to vasodilation, vasoconstriction occurs as a result of the increase in intracellular Ca^{2+} within VSMCs (Scotland et al., 2001). Vasoconstriction is important for thermoregulation and for the prevention of blood loss caused by haemorrhage. A broad range of environmental, mechanical and chemical stimuli also regulates vasoconstriction. A drop in ambient temperature, stretching of skeletal muscle or increases in the concentration of circulating oxygen, for instance, can lead to vasoconstriction. Amongst the intrinsic factors, however, the most common involve hormonal and paracrine control. These comprise mainly the secretion of epinephrine, norepinephrine, angiotensin II (Ang-II) and endothelin-1 (ET-1). Epinephrine and norepinephrine are secreted by the adrenal gland and sympathetic nerve terminals, respectively (Minneman, 1988; Loesch et al., 1997). Ang-II in turn, is the product of two cleavages on angiotensinogen, a hormone secreted mainly by the liver. Angiotensinogen is first converted to angiotensin I and later to Ang-II by the angiotensin-converting enzyme (ACE). ET-1, on the other hand, is a potent vasoconstrictor released by the EC. The hormones that mediate vasoconstriction act through specific G protein-coupled receptors located in the VSMCs or EC. Binding of these hormones to their receptors activates phospholipase C and the subsequent production of inositol 1,4,5-trisphosphate (IP3). All signal transduction pathways

converge to increase the intracellular concentration of Ca^{2+}. IP3 signals the release of Ca^{2+} from the endoplasmic reticulum by binding to receptor-gated Ca^{2+} channels. Ca^{2+} then binds calmodulin, which in turns phosphorylates myosin light heavy chain promoting smooth muscle contraction (Carl et al., 1996).

Ageing

Ageing is the inevitable consequence of the functional decline experienced with time. In fact, ageing is a major risk factor for the development of common degenerative conditions such as Alzheimer's, diabetes, cancer and CVD. Advances in medicine have successfully been translated into a longer life expectancy. For humans in developed countries, life expectancy now extends beyond 80 years (Aunan et al., 2016).

Ageing is a very complex, multifactorial process. Ageing is characterized by the loss of cellular fitness caused by the accumulation of damage and the inability to repair such damage (López-Otín et al., 2013). At the molecular level, ageing could be understood as the consequence of the following features: genomic instability, epigenetic alterations, impaired protein homeostasis, deregulated nutrient sensing, cellular senescence, stem cell exhaustion, mitochondrial dysfunction, and altered intercellular communication. A thorough review of these mechanisms is beyond the aim of this introduction. However, for a detailed description the reader is referred to

the following reviews (López-Otín et al., 2013; Moskalev et al., 2013; Aunan et al., 2016).

The age-related accumulation of genomic damage includes aberrant DNA methylation, chromosomal aneuploidy, telomere exhaustion, transcriptional noise, and epigenetic alterations such as posttranslational histone modifications and chromatin remodelling. Aneuploidy, for instance, is the gain or loss of whole chromosomes. It is caused by faulty checkpoints during cell division and has been associated with degenerative condition such as cancer and ageing (Faggioli et al., 2012). Telomere exhaustion is another example that clearly demonstrates how damage to DNA will accelerate ageing. DNA polymerase is not able to replicate the terminal ends of DNA strands. Hence, upon replication, chromosomes are exposed to deterioration or become susceptible to fuse with other chromosomes giving the cell a limited capacity to replicate. Clearly, damage to DNA will result in defective proteins and signalling pathways that once associated with an already compromised capacity of the cell to repair itself (see below) will exacerbate the ageing process (Henriques and Ferreira, 2012).

Eukaryotic cells make use of protein quality control mechanisms that guarantee appropriate protein folding as well as organelle repair and renewal. These involve the chaperone-mediated protein folding mechanisms and the degradation of damaged proteins via the proteasome and the lysosome. The controlled degradation of

misfolded proteins prevents the cellular dysfunction caused by accumulated damage. The capacity of the cell to initiate protein degradation diminishes with age. Evidently, this leads to the accumulation of protein aggregates, damaged organelles and cellular dysfunction (Mizushima et al., 2008; Powers et al., 2009).

A significant body of evidence supports the notion that caloric restriction extends lifespan in mammals and other organisms (García-Prieto and Fernández-Alfonso, 2016; Lee and Longo, 2016). Part of the mechanisms by which caloric restriction extends lifespan involves the insulin-like growth factor 1 (IGF-1) and insulin signalling pathways, which regulate glucose uptake. It has been demonstrated that down-regulation of these pathways by mutations that reduce the expression of receptors for either IGF-1 or insulin are associated with longevity (Barzilai et al., 2012; López-Otín et al., 2013). The reason behind this is that cells with lower rates of growth and metabolism also experience less cumulative damage. IGF-1/glucose signalling naturally decreases with age as a compensatory mechanism to prevent damage and extend life. However, significant lower sensitivity to nutrient signalling is not compatible with life and as such, the age-related, compromised IGF-1/glucose signalling eventually accelerates ageing (López-Otín et al., 2013).

Cell senescence refers to the incapacity of a cell to grow and/or divide. This is caused mainly by telomere shortening and non-telomeric DNA damage. Cell senescence is not necessarily a deleterious process. Instead, it might represent a survival

mechanism that prevents the proliferation of dysfunctional cells. When coupled to the adequate recruitment of progenitor cells, organelles and cells are renewed and the tissue is able to adapt. However, with age the regenerative capacity of progenitor cells fails. Hence, the accumulation of senescent cells aggravates the age-related functional decline (Matheu et al., 2009; Baker et al., 2011).

Ageing has been attributed to an increased production of reactive oxygen species (ROS) mainly by dysfunctional mitochondria and NADHP oxidases (LeBlanc and Hoying, 2016). As mentioned above, the accumulation of protein aggregates and damaged organelles (including mitochondria) eventually leads to cell death. ROS play important signalling functions within cells. However, similar to anabolic and nutrient signalling downstream pathways, beyond a certain threshold overproduction of ROS becomes deleterious and contributes to a self-perpetuating loop of organelle damage and cellular dysfunction (LeBlanc and Hoying, 2016).

Finally, ageing is also the consequence of compromised cellular communication. As expected, the accumulation of damaged cells with age will eventually lead to defective tissues and a reduced capacity of the organism to function as a whole. The main characteristics of ageing at the systemic level comprise immunosuppression and higher levels of inflammation. The inability to maintain haematopoiesis at an advanced age, for instance, results in a diminished capacity of the immune system to clear pathogens and senescent cells. These cells in turn, tend to secrete more pro-

inflammatory cytokines, which uncontrolled production is particular to the development of insulin resistance and obesity, degenerative conditions associated with ageing (López-Otín et al., 2013). Figure 5 summarizes the common molecular features of ageing.

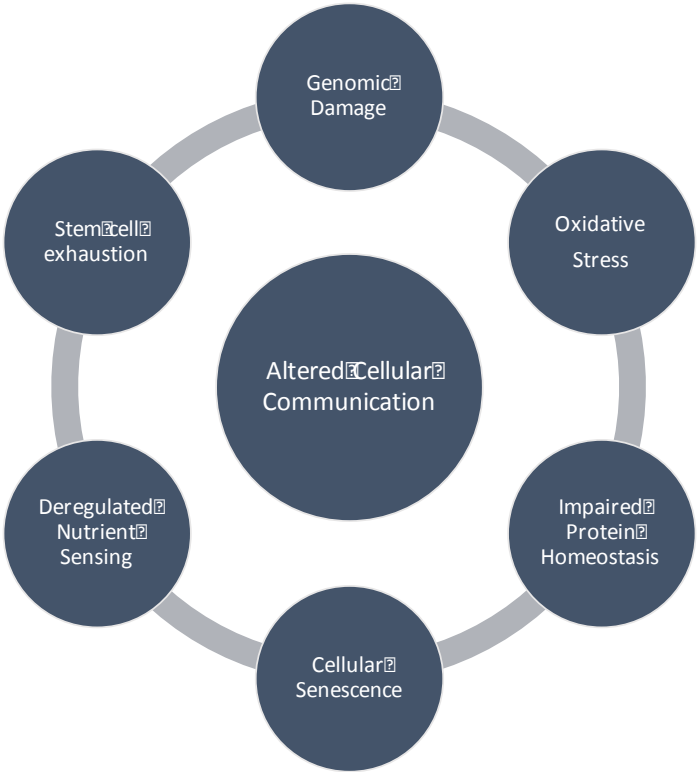

FIGURE 5. MOLECULAR, BIOCHEMICAL AND CELLULAR FEATURES OF AGEING.

Adapted from (López-Otín et al., 2013).

Ageing and cardiovascular disease

The broad molecular and biochemical features mentioned above translate, amongst other conditions, into CVD. Even though CVD can progress independently of ageing, the latter is the most important risk factor for its development. Collectively, CVD refers to pathological states of the blood vessels and the heart. It occurs at the level of the EC, VSMCs and the extracellular matrix of blood vessels and includes hypertension, arthrosclerosis, stroke, poor tissue perfusion, increased arterial thickness and endothelial dysfunction (North and Sinclair, 2012; Rubio-Ruiz et al., 2014). Clearly, each tissue in the body will present a distinct physiological (and degenerative) age-related profile. In particular, the ageing vasculature is characterized by a positive feedback loop of higher levels of oxidative stress, a reduced bioavailability of NO, an imbalanced production of vasodilators and vasoconstrictors, and a pro-inflammatory environment (Rubio-Ruiz et al., 2014).

Within the aged EC, the high production of the $O_2^{\cdot -}$ is critical for the pathogenesis of endothelial dysfunction. This is mainly because of how $O_2^{\cdot -}$ interferes with NO signalling. With ageing, there is a natural increase in the production of $O_2^{\cdot -}$ by mitochondria and NADPH oxidases. $O_2^{\cdot -}$ can scavenge NO to form peroxynitrite ($ONOO^-$). Interestingly, $ONOO^-$ can oxidize tetrahydrobiopterin (BH_4), a co-factor of eNOS. In the absence of BH_4, eNOS becomes uncoupled and produces $O_2^{\cdot -}$, which leads

to a self-perpetuating cycle of oxidative stress and lower bioavailability of NO (Blackwell et al., 2004). Further, ageing is characterized by a higher activity of arginase, the enzyme responsible for the breakdown of L-arginine. As mentioned above, L-arginine is necessary for the production of NO. As a consequence of less L-arginine available, less NO is synthetized in the EC (Santhanam et al., 2008). Given the role of NO in promoting vasodilation, and preventing platelet aggregation and leukocyte adhesion to the endothelium, it is no surprise that the reduced bioavailability of NO is one of the earliest indicators of CVD and it is associated with the development of hypertension and arthrosclerosis (Torregrossa et al., 2011).

Ageing is also associated with a decline in the signalling of EDHFs and PGI_2. It has been shown that long-term inhibition of ACE recovered the EDHF-mediated dilator responses in the mesenteric arteries of spontaneously hypertensive aged rats (Goto et al., 2004). Further, with age there is a greater production of vasoconstrictors such as ET-1, Ang II and thromboxane A_2 (Long et al., 2005; Versari et al., 2009). The shift in balance towards a greater production of vasoconstrictors also promotes a pro-inflammatory environment. Both thromboxane A_2 and PGI_2 are derivatives of the arachidonic acid pathway. Hence, thromboxane-A synthase (the enzyme that produces thromboxane A_2) and PTGIS compete for substrates for their respective synthesis. Not only does thromboxane A_2 mediates vasoconstriction, it also activates platelets and promotes inflammation (McClelland et al., 2009). Vascular inflammation seems to be the ultimate manifestation of CVD. Interestingly, all hallmarks of ageing

can be mirrored under pro-inflammatory environments. That is, oxidative stress, a reduced bioavailability of NO, a prevalence of vasoconstrictors over vasodilators and higher rates of apoptosis can be elicited in young rat carotid arteries when treated with tumour necrosis factor- α (TNF-α) (Csiszar et al., 2007).

In the aged blood vessels, CVD is also characterized by morphological and functional alterations of VSMCs and the extracellular matrix. Ageing has been associated with a proliferative state of VSMCs, which is caused by a greater activity of c-fos and the consequent elevation in the expression of cyclin A (Rubio-Ruiz et al., 2014), the overexpression of platelet derived growth factors (Yang et al., 2009), and higher sympathetic drive to the vessel (Schiattarella et al., 2014). Clearly, an uncontrolled proliferative state of VSMCs aggravates conditions such as atherosclerosis (Moon et al., 2001). These changes are accompanied by an increased constriction responses to ET-1 and potassium chloride as demonstrated in the carotid arteries of aged mice (Donato et al., 2009) and the aorta of aged rats (Rubio et al., 2006), respectively. In addition to this, cGMP signalling in the VSMCs falls in similar fashion as NO bioavailability in EC. This leads to a reduced activation of protein kinase G-1 (PKG-1) and hence, the inability to fully relax (Lin et al., 2001).

The extracellular matrix offers a scaffold necessary for the appropriate functioning of the smooth muscle and the endothelium. During ageing, the balance amongst the structural components of the extracellular matrix such as elastin, collagen,

proteoglycans and glycoproteins is disrupted. This is mainly due to the overexpression of matrix metalloproteinases and activation of zymogens. The aged extracellular matrix becomes fibrotic and non-compliant. In fact, a fibrotic extracellular matrix is one of the consequences and most serious adverse effects of hypertension. Constant increases in BP results in an extracellular matrix unable to accommodate the mechanical demands of the smooth muscle. Therefore, vascular damage such as stroke and aneurysm occurs (Rubio et al., 2006). Figure 6 shows the particular features that accompany CVD in the aged blood vessel.

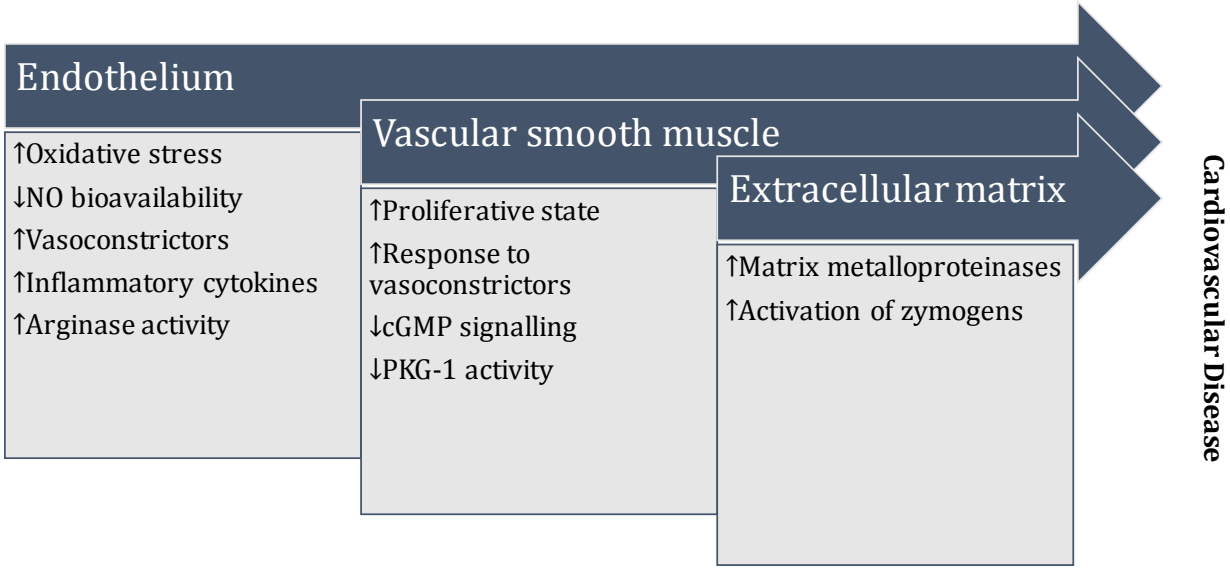

FIGURE 6. BIOCHEMICAL FEATURES OF THE AGED BLOOD VESSEL THAT UNDERLIE THE DEVELOPMENT OF CARDIOVASCULAR DISEASE.

Nitric oxide (NO); Cyclic guanosine monophosphate (cGMP); Protein kinase 1 (PKG-1) (author generated).

Nutraceutical interventions in cardiovascular disease

Cardiovascular disease was the second most common cause of death in the UK in 2014 with approximately 155.00 deaths (CDS 2015). Clearly, the socio-economic burden of CVD is significant. Even though conditions such as hypertension can to some degree, be safely and effectively managed with prescription medication in the elderly (Morgenstern and Byyny, 1992), there is a considerable need for lifestyle modifications such as physical exercise and nutraceutical supplementation that lower the incidence or help ameliorate the severity of CVD. Nutraceuticals are food or food supplements with naturally occurring compounds known to have positive effects on health. From the socio-economic perspective, nutraceutical supplementation offers substantial benefits over traditional pharmaceutical products due its relatively low costs and health risks, and the less time required to produce and approve (Seals et al., 2014). In this regard, extensive attention has been given during the last decade to antioxidant supplementation. Considering the arguments above, there is reason to believe that by restoring oxidative balance within the aged EC, endothelial function could be preserved. Thus far, some antioxidants have been found to improve cardiovascular parameters in the elderly. For instance, in a recent clinical trial, consumption of an antioxidant cocktail consisting of vitamin C, vitamin E and lipoic acid, significantly improved flow-mediated dilation (FMD) of the brachial artery in elderly subjects. This was attributed to increases in the levels of superoxide

dismutase, total antioxidant capacity as well as reductions in thiobarbituric acid reactive substances (Wray et al., 2012).

Resveratrol is a naturally occurring polyphenol and phytoalexin found mainly in berries, peanuts and the skin of red grapes. RV bears significant antioxidant and anti-inflammatory properties. The interest in compounds derived from red grapes originated after the observation of an inverse correlation between the consumption of red wine and the frequency of CVD (Rimm et al., 1996). Even though it was proven that ethanol on its own offers some cardiovascular protection by means of increasing the circulating levels of high-density lipoprotein, a more thorough analysis of red wines showed that RV was responsible for a reduction in serum lipids (Siemann and Creasy, 1992).

Resveratrol is a versatile molecule with many cellular targets. The major cardiovascular benefits of RV include the inhibition of lipid peroxidation, chelation of copper, scavenging of free radicals, alteration in the synthesis of eicosanoids, inhibition of platelet aggregation, anti-inflammatory activity, modulation of lipid metabolism, promotion of autophagy and improvements in NO bioavailability and vasodilation (Bhat KPL et al., 2001; Vang et al., 2011). Thus far, the majority of the studies that support the health benefits of RV has been conducted *in-vitro*. However, proof of such potential has been difficult to obtain *in-vivo*. Part of the disagreement between the two models is attributed to RV metabolism in living organisms. *In-vivo*,

the effects of RV, or any other nutraceutical for that matter, might be caused not by RV itself, but by products of its metabolism. Characterization of such metabolites is critical in the understanding and treatment of CVD. One recent report in nutrition research stated that data gathered from scientific studies on nutraceutical supplementation might not be physiologically relevant because nonspecific results, those caused by unknown metabolites of the original compound used, cannot be precisely related to health outcomes (Harnly, 2016). The methodology used, for instance, might be inappropriate for the determination of redox status since commonly used reducing agents are interferents. Further, if the exact active compound is unknown, it is not possible to target putative cellular mechanisms underlying the onset and development of the disease (Harnly, 2016). The concentration of RV and duration of treatment of cells/tissue *in-vitro* are not easily reproduced *in-vivo*, especially when RV is given orally (Walle, 2011). The absorption of RV in humans is very high. Absorption was reported to be at least 70% after oral consumption of 25 mg of RV. This leads to fairly high concentrations of RV metabolites in plasma (2 μM) with a half-life of approximately 9.5 hours but only trace amounts of non-metabolized RV (<10 ng·mL) (Walle et al., 2004). Hence, it should not come as a surprise that only a handful of studies have demonstrated efficacy of RV *in-vivo*. However, this highlights the necessity of characterizing the stability and bioavailability of RV when tested either *in-vivo* or *ex-vivo*. Otherwise, If RV is not stable or available under experimental conditions the data gathered would be rendered rather irrelevant and pose little translational potential, as it has been suggested previously (Gescher and Steward, 2003). That said, once the stability and

bioavailability are demonstrated and the appropriate doses and duration of treatment determined, there is reason to believe that supplementation with RV would offer significant potential alleviating the socio-economic burden of CVD, particularly in the elderly.

Evidence attributing positive effects of RV to the vasculature is fairly recent. A thorough review on the molecular, biochemical and physiological effects of RV on aged vessels is presented in the Publications section of the Appendix. However, regarding the precise effects of RV on endothelial function, *ex-vivo* RV is reported to induce concentration-dependent dilation of isolated mesenteric and uterine arteries in rats and guinea pigs, respectively (Naderali et al., 2000, 2001). Further, chronic oral supplementation with RV has been shown to induce concentration-dependent dilation in mesenteric arteries and the aorta of obese Wistar and spontaneously hypertensive rats (SHR) (Naderali et al., 2001; Rush et al., 2007, 2007; Bhatt et al., 2011). Such improvements in vasodilation have been attributed to a reduction in levels of oxidative stress and higher expression and activity of eNOS (Bhatt et al., 2011). In hypertensive and overweight humans, both acute and chronic supplementation with RV led to significant improvements in FMD of the brachial artery (Wong et al., 2011; Wong, Berry, et al., 2013). These findings have been challenged by a recent series of publications in which it was demonstrated that RV blunts important metabolic and physiological adaptations critical for vascular health. For instance, *in-vitro* acute exposure to RV inhibits AMPK activity, fatty-acid oxidation

and glucose metabolism in human myotubes (Skrobuk et al., 2012). Further, *in-vivo* chronic supplementation with RV decreased the levels of PGI_2 while increased the levels of thromboxane synthase in the vastus lateralis of exercising elderly men. RV also abolished the positive effects of exercise on low-density lipoprotein, total cholesterol/high-density lipoprotein ratio and triglyceride concentrations in blood (Gliemann et al., 2013). In line with these findings, chronic supplementation with RV did not change plasma lipids, or inflammatory markers in adult non-obese women with normal glucose tolerance (Yoshino et al., 2012).

Aims and hypothesis

The studies that comprise this thesis aimed to better understand the effect of RV on endothelial function during ageing. There is a murine and a human component to these studies. The aim of the murine component is to establish the vasodilator response of the isolated pressurized mouse femoral artery to pharmacological and mechanical stimuli following incubation with RV. The human component aims to determine the effects of supplementation with RV on endothelial function and oxygen consumption in coronary artery disease elderly patients. Hence, the animal study aimed to provide mechanistic insight on the vascular and physiological parameters assessed in humans. The particular aims were to:

1. Establish the vasodilator responses of femoral arteries from young (4 months), and aged (26 months) mice to pharmacological and mechanical stimuli, *ex-vivo*;
2. Characterize the endothelium-dependent vasodilator components of the femoral artery from young (4 months), and aged (26 months) mice, *ex-vivo*.
3. Determine the stability of RV during experimental settings of pressure myography;
4. Determine to what extent acetylcholine-induced and FMD of the femoral artery from young (4 months), and aged (26 months) mice can be improved with RV, *ex-vivo*
5. Determine the effects of RV on the production of NO by cultured EC in response to acetylcholine and laminar flow;
6. Determine to what extent FMD and oxygen consumption can be improved with RV in the brachial artery of coronary arterial disease elderly patients.

We hypothesised that acute treatment with high doses of RV would improve dilation of the isolated pressurized femoral and brachial arteries of aged mice and coronary artery disease elderly patients, respectively.

Chapter 2

Characterization of the age-related endothelium-dependent dilator responses of isolated mouse femoral arteries to acetylcholine and intraluminal flow

Abstract

Ageing is a major risk factor for the development of endothelial dysfunction and cardiovascular disease. Here, we investigated the effects of ageing on the endothelium-dependent dilator responses of the isolated mouse femoral artery. Segments from 4- (young) and 26-month-old (old) C57BL/6 male mice were isolated after sacrifice and mounted on a pressure myograph. Dilator responses to increasing doses of acetylcholine (ACh, 10^{-9} - 10^{-3} M) and intraluminal flow (5-10 μL·min^{-1}) were determined in pre-constricted vessels (phenylephrine 10^{-5} M) in the presence of the inhibitors L-NG-nitro-L-arginine (LNNA, 100 μM), indomethacin (10 uM), or apamin (100 nM) + Tram 34 (1 uM). Maximal dilator responses to ACh and flow were preserved with age (n.s.). We observed no significant effects of inhibitors on ACh-induced dilation (n.s.). However, LNNA or apamin + Tram 34 attenuated FMD in both groups ($p<0.05$). Only in the old was FMD reduced in the presence of indomethacin ($p<0.05$). Our findings suggest important age-related effects on the mechanisms regulating arterial tone despite preserved degrees of relaxation.

Key words: Vasodilation; Nitric Oxide, Flow-mediated Dilation; EDHFs, Prostaglandin.

Introduction

The vascular endothelium is a monolayer of cells that lines the inner wall of the blood vessels. It is critical for the regulation of vascular tone as it is responsible, amongst other functions, for modulating the constriction and dilation of the vessels in response to a broad variety of chemical and mechanical stimuli. Endothelium-dependent vasodilation is an intricate process. It depends on the interplay of at least three (types of) molecules; nitric oxide (NO), prostacyclin (PGI_2), and endothelial-derived hyperpolarizing factors (EDHFs), such as hydrogen peroxide (H_2O_2), potassium ions (K^+) and products of cytochrome p450 enzymes (Luksha et al., 2009; Hangaard et al., 2015; Yang et al., 2015). The predominance that each molecule plays in dilation is highly dependent on the species, vascular bed, vessel size and nature of the stimulus (Clifford et al., 2010; Freed et al., 2014). However, to a certain degree, they all contribute to dilation and are necessary for an appropriate functioning of the endothelium. Mechanical forces, such as shear stress and agonists such as acetylcholine (ACh) increase vessel diameter by stimulating the production of dilator factors such as NO, PGI_2 and EDHFs. As explained in the previous chapter, these substances then elicit smooth muscle relaxation through the intracellular increase in cGMP, cAMP or membrane hyperpolarization, respectively (Durand and Gutterman, 2013).

Compromised endothelial function is characterized by a decreased capacity of the blood vessels to dilate in response to vasodilator stimuli, and has been linked to the development of cardiovascular disease (CVD) (Ng et al., 2015). It is generally accepted that ageing is associated with a decline in endothelial function. In fact, some consider ageing to be the major risk factor for the development of CVD (North and Sinclair, 2012). The aged vasculature has been associated with a reduced bioavailability of NO (Strait and Lakatta, 2012). An age-related reduced endothelium-dependent dilation to ACh and intraluminal flow has been reported previously in the aorta, mesenteric and skeletal muscle feeding arteries (Zhou et al., 2010; Donato et al., 2011:1; Sindler et al., 2011; Trott et al., 2011; Fleenor et al., 2013). However, there is significant heterogeneity in the vasculature and a growing body of evidence supports the notion that some vascular beds, such as the aorta or arteries in the upper limbs, are less prone to develop compromised endothelial function than arteries in the lower limbs, such as the femoral artery (Newcomer et al., 2004; Wray et al., 2006). Whereas in humans this could be due to differences in hydrostatic forces in the proximal and distal vasculature related to the upright posture (Dinenno et al., 2000; Pawelczyk and Levine, 2002; Newcomer et al., 2004), in small rodents it might also be related to the distribution and expression of α1-adrenergic receptors in certain vascular beds and the consequent hypertrophy of smooth muscle caused by sustained elevations in adrenergic stimulus (Pauletto et al., 1991; Giangrande et al., 2007; Cipolletta et al., 2010; Gradinaru et al., 2015). However, thus far conflicting reports have been published regarding the effects of ageing on endothelium-dependent dilation of arteries of the lower limbs and some studies have shown that in rodents,

compromised endothelial function in these vascular beds is associated with a decreased bioavailability of NO and eNOS activity (Bearden et al., 2004; Baron et al., 2014; Sinkler and Segal, 2014; Jelinic et al., 2015). This line of enquiry is important because the skeletal muscle circulation plays a critical role in the regulation of BP (Newcomer et al., 2008). Vascular resistance, as determined by the diameter of the resistance arteries and arterioles in the periphery and to some degree conduit arteries too, is directly related to BP when cardiac output is constant (O'Rourke and Hashimoto, 2007; Newcomer et al., 2008). Further, it has been demonstrated that peripheral arterial disease progresses from the arteries in the lower extremities to the upper vasculature (Newcomer et al., 2004). Finally, a molecular network between the blood vessel and the skeletal muscle has already been reported. It is assumed that by increasing the delivery of oxygen and nutrients to the muscle, the capacity of the muscle to grow and adapt to mechanical and nutritional stimuli would also increase (Arsic et al., 2004; Wagatsuma et al., 2006; Messina et al., 2007). Consequently, by improving endothelial function of the arteries feeding the skeletal muscle, other age-related degenerative conditions such as sarcopenia can be targeted.

The anatomical heterogeneity in the development of the age-related endothelial dysfunction and the pathological conditions associated with it, such as arteriosclerosis, might be partially explained by local changes in the secretion and availability of vasodilators over time (Matz et al., 2000; Puzserova et al., 2014). Previous studies have reported that the NO-dependent component of the ACh-

induced dilation of the femoral artery of Wistar Kyoto (Puzserova et al., 2014) and Sprague Dawley (Shi et al., 2008) rats decreases with age despite a preserved overall dilator response. Such change is associated with a more predominant role of PGI_2 in dilation (Shi et al., 2008). Contrary to this, the NO-dependent components of dilation in the femoral artery of piglets become of greater importance after birth (Støen et al., 2001). In first order arterioles of Wistar Kyoto rats, maximal dilation in response to intraluminal flow increases from 4 to 12 weeks of age due to an enhanced secretion of NO and PGI_2 (Koller and Huang, 1999). However, with advanced ageing the opposite trend has been reported. Fischer 344 rats show lower levels of dilation of the soleus feed arteries in response to intraluminal flow due to a decreased production of both NO and PGI_2 when compared to young controls (Woodman et al., 2003). Unfortunately, these studies are only a handful and little information is available regarding the potential effects of ageing on the qualitative endothelium-dependent mechanisms mediating dilation of the femoral artery.

Pressure myography allows for the in-depth characterization of the intrinsic responses to pharmacological and mechanical stimuli of isolated vessels. Because it maintains vessels in near physiological conditions in regards to temperature, oxygen and pressure, it allows for the characterization of responses to individual stimuli without the complicating issues of compensatory mechanisms present *in-vivo.* Hence, it provides a useful tool for the study of most of the *in-vivo* properties of isolated vessels and to some degree, the responses can be extrapolated to the authentic

behaviour of the vascular bed *in-vivo* (Shahid and Buys, 2013). Here, we employed pressure myography to determine the effects of ageing on dilation of the isolated pressurized mouse femoral artery to increasing doses of ACh and intraluminal flow. We also determined the NO-dependent and –independent components of the endothelium-mediated dilation of the artery. The particular aims of this study were to 1) establish the vasodilator responses of femoral arteries from young (4 months), and aged (26 months) mice to pharmacological and mechanical stimuli, and 2) characterize the endothelium-dependent vasodilator components of the femoral artery from young (4 months), and aged (26 months) mice, *ex-vivo.* We hypothesised that in femoral arteries 1) a significant age-related compromised endothelial function develops and 2) that such compromised endothelial function would be due to changes in the release or predominance that endothelial factors play in dilation.

Methods

To characterize vasodilator responses, 4- (young) and 26-month-old (old) pathogen-free male C57BL/6 mice were humanely euthanised in accordance with the 'Animals (Scientific Procedures) Act 1986' and Institutional guidelines (ethics reference number SE131413). The animals were obtained from Charles Rivers Laboratories (UK) where they were maintained until one week before the experiments. The body mass was 28 ± 1 g in young and 30 ± 1 g in old animals ($p<0.05$). Animals were killed by cervical dislocation. The left leg of each mouse was removed and pinned on a clear

glass dissecting dish containing a Sylgard silicon base in ice-cold physiological salt solution (PSS) [composition [mM]: 119 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.1 $KHPO_4$, 0.03 K_2EDTA, 5.5 glucose, 1.6 $CaCl_2 \cdot 2H_2O$, pH 7.4] (Figures 7 A-B). Segments of the femoral artery (proximal location near the groin in the area close to the inguinal ligament; ~3 mm in length) were finely dissected (the fat tissue layer and connective tissue surrounding each vessel were also removed) (Figures 7 C-E) and mounted between two glass cannulae on a pressure myograph chamber (Living Systems Instrumentation, Burlington, VT, USA) (Figures 7 F-H). The preparation was checked for leaks and readjusted if required. Arteries were initially pressurised to an intravascular (luminal) pressure of 60 mmHg (Shahid and Buys, 2013) and maintained at that pressure using a pressure servo-control unit (Living Systems, Burlington, USA). The arteries were equilibrated and constantly superfused in a continuous source of PSS at 37℃, pH 7.4, and gassed with 95% air-5% CO_2, with the superfusate entering and leaving the chamber via luer connections on the side of the chamber. The chamber was placed over an inverted microscope (Nikon Eclipse TS100, Japan) to constantly measure the lumen and internal diameters of the vessel using a video dimension analyser, with data recorded on a computer using Chartlab 5 software (Powerlab system, AD Instruments, UK) (Figures 7 I-J) for later analysis. The viability of the femoral arteries was assessed for their ability to constrict in response to a high potassium physiological salt solution (KPSS; composition [mM]: 119 NaCl, 60 KCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.17 $KHPO_4$, 0.03 K_2EDTA, 5.5 glucose, 1.6 $CaCl_2 \cdot 2H_2O$; pH 7.4). In preliminary experiments, we determined constrictor responses to increasing doses of phenylephrine (Phe; 10^{-9} – 10^{-3} M). We obtained optimal

constriction (80%) upon perfusion of the arteries with Phe 10^{-5} M (Shahid and Buys, 2013) (Appendix; Supplementary Data Figure 33). Therefore, Phe 10^{-5} M was used for all further experiments to pre-constrict vessels. We observed no development of myogenic tone in the arteries after an equilibration period of one hour in PSS with intraluminal pressure of 60 mmHg (Appendix; Supplementary Data Figure 34).

Characterization of the dilator response

The arteries were pre-constricted with Phe 10^{-5} M and were left to stabilize. After attainment of a stable level of constriction, dose responses to ACh (10^{-9}-10^{-3} M) (Shahid and Buys, 2013) were constructed. The arteries were washed with PSS and pre-constricted again with Phe 10^{-5} M to determine dilator responses to intraluminal flow (5-10 $\mu L.min^{-1}$). These flow rates induced optimal dilation without disrupting endothelial function (Sun et al., 2001) (Appendix; Supplementary Data Figure 35).

To investigate the contribution of known vasodilators to ACh-induced and flow-mediated dilation (FMD) of the isolated pressurized mouse femoral artery we incubated segments of the artery with L-NG-nitro-L-arginine (LNNA, an inhibitor of nitric oxide synthase; 100 uM), indomethacin (a nonselective inhibitor of cyclooxygenases; 10 uM), or the combination of apamin (a selective blocker of small-conductance calcium-activated potassium channels; 100 nM) and Tram 34 (a

selective blocker of intermediate-conductance calcium-activated potassium channels; 1 uM). The concentration of the drugs used was based on previous characterization studies on endothelium-dependent dilation of isolated arteries (Støen et al., 2003; Gauthier et al., 2011; Leo et al., 2011; Han et al., 2013; Morton et al., 2015). The drugs were applied intraluminally for 30 minutes using a 1-mL syringe inserted into one end of the 3-way luer connection on the side of the pressure myograph chamber while incubated in PSS. For control vessels, PSS was applied instead. After the 30-minute incubation, dilator responses in the presence of inhibitors, to ACh (10^{-9}-10^{-3} M) and intraluminal flow (5-10 $\mu L \cdot min^{-1}$) were determined. Intraluminal flow was introduced for 4-5 minutes at each flow rate with a peristaltic pump (flow control pump FC, Living Systems Instrumentation, Burlington, VT, USA) connected to the right cannula (distal end of the vessel) of the chamber. After 4-5 minutes, intraluminal flow was stopped and the vessel kept at an intramural pressure of 60 mmHg. The pressure gradient within the vessel was constantly monitored with a PM/4 perfusion pressure monitor (Living Systems Instrumentation, Burlington, VT, USA) connected to both the proximal and distal pressure transducers. The system automatically adjusts the distal pressure to compensate for changes in proximal pressure with flow. Hence, the pressure within the vessel was kept constant at 60 mmHg. At the end of each experiment, the arteries were superfused with calcium (Ca^{2+})-free PSS containing 2 mM EGTA for 20 minutes to obtain passive diameters. Unless otherwise stated, each group of experiments consisted of at least five animals (1 vessel per animal). All drugs were obtained from Sigma-Aldrich (Poole, UK). The percentage of the dilator response to ACh and intraluminal flow was calculated as:

$$\frac{\text{(Dilation – Constriction to Phe } 10^{-5}\text{ M)}}{\text{(Resting Diameter – Constriction to Phe } 10^{-5}\text{ M)}} \times 100$$

We noticed two main components to FMD; maximal initial FMD and sustained FMD. In response to intraluminal flow, maximal dilation was not maintained for the entirety of the 4-5 minutes that flow was introduced. In general, the arteries displayed a maximal degree of dilation followed by a transient plateau (sustained dilation) before auto-regulating their diameters to stable levels almost identical to the pre-constricted state. In addition to the initial flow-mediated maximal dilation, we calculated the diameter during the plateau, stable phase and prior cessation of flow. The variation in diameter for both phases was normalized to resting diameter as in the formula above. Wall shear stress was calculated from:

$$\tau = 4\,\eta Q/\pi r^3$$

where τ is shear stress (in $dyn \cdot cm^{-2}$), η is viscosity (0.007 $dyn \cdot s \cdot cm^{-2}$), Q is flow (in $mL \cdot s^{-1}$) and r is radius (in cm) (Azzawi and Austin, 2007).

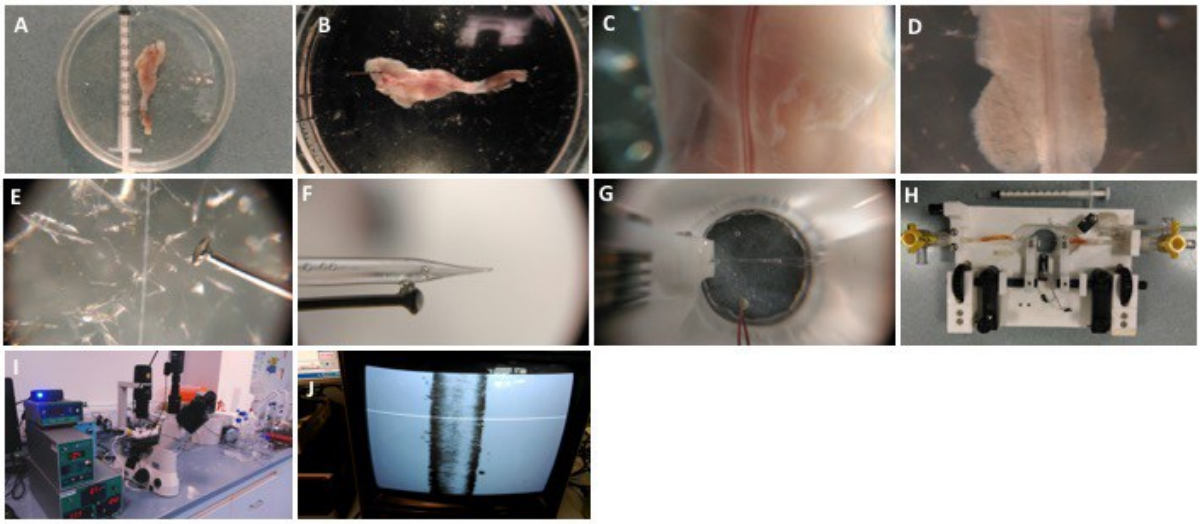

FIGURE 7. DISSECTION OF THE MOUSE FEMORAL ARTERY AND CHARACTERIZATION OF ITS DILATOR RESPONSES BY MEANS OF PRESSURE MYOGRAPHY.

The left leg of each mouse was removed and pinned on a clear glass dissecting dish containing a Sylgard silicon base in ice-cold PSS (A and B). Segments of the femoral artery (proximal location near the groin in the area close to the inguinal ligament; ~3 mm in length) were finely dissected (the fat tissue layer and connective tissue surrounding each vessel were also removed) (C - E). Figures E and F also show a steel dissection pin (diameter 0.5 mm) for size reference. The dissected segment of the mouse femoral artery was mounted between two glass cannulae on a pressure myograph chamber (G and H). The internal diameter of the vessel was monitored using a video dimension analyser, with data recorded on a computer using Chartlab 5 software (I-J).

Statistical Analysis

The dilator responses elicited by ACh and intraluminal flow are expressed as percentage relaxation corresponding to the increase from the pre-constricted value. The Shapiro-Wilk test was used to assess the conformity of the data to normal distribution. Given normal distribution, concentration response curves were assessed using three-way repeated-measure analysis of variance (ANOVA), with subsequent unpaired comparisons where needed, to determine the main effects of treatment (dilator stimulus), age, condition (inhibitor), and the interaction between them. Values of $p<0.05$ were considered significant. Data are represented as mean ± standard error of mean (SEM). Power calculations were not conducted *a priori* for this study.

Results

Resting diameters and constrictor responses to KPSS and Phe

The arteries used in these studies had resting diameters of 139 ± 46 μm and 150 ± 34 μm for the young (n = 11) and old (n = 6) mice, respectively. We observed no significant effect of ageing on lumen diameter. The degree of constriction to KPSS

(young: 49 ±10 μm *vs.* old: 31 ± 15 μm) and Phe 10^{-5} M (young: 49 ±15 μm *vs.* old: 31 ± 15 μm) did not differ significantly between groups. Figure 8 shows the constrictor responses to KPSS and Phe 10^{-5} M of segments of the femoral artery from young and old mice.

Effects of age on the dilator response to ACh and intraluminal flow

ACh (10^{-9}-10^{-3} M) induced concentration-dependent dilation in isolated pressurized femoral arteries from young and old mice. However, we observed no significant difference in ACh-induced dilation of the isolated pressurized mouse femoral artery with ageing (Figure 9). Intraluminal flow resulted in dilation of arteries from both young and old mice. Similar to ACh-induced dilation, maximal initial FMD was preserved with age (Figure 10). Wall shear stress values of the isolated pressurized femoral artery as a consequence of intraluminal flow are presented in Table 1. Mean shear stress values were higher in the old in response to intraluminal flow rates of 5 and 8 $\mu L.min^{-1}$.

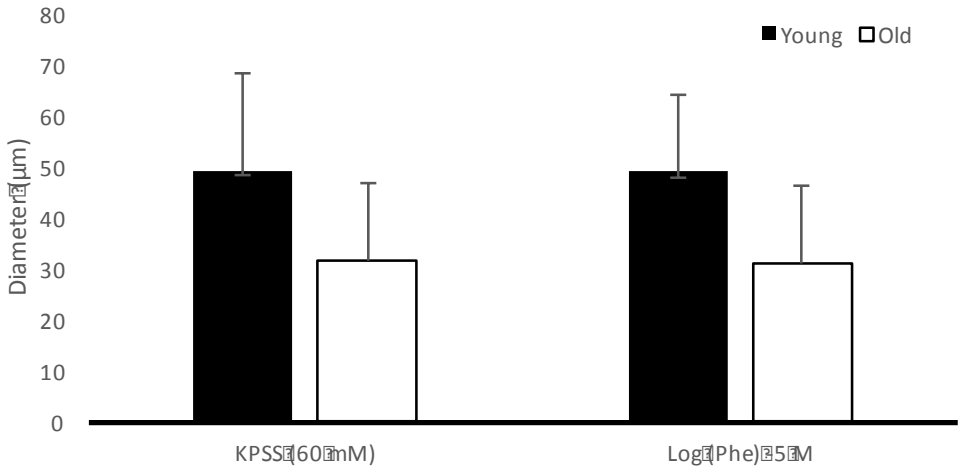

FIGURE 8. CONSTRICTOR RESPONSES TO KPSS AND PHE.

KPSS (60 mM)- and Phe (10^{-5} M)-induced constriction of the isolated pressurized femoral artery from 4-(young; *n* = 11) and 26-month old mice (old; *n* = 6). We observed no statistical differences in KPSS- or Phe-induced constriction between the arteries of young and old mice. Data are means ± SEM.

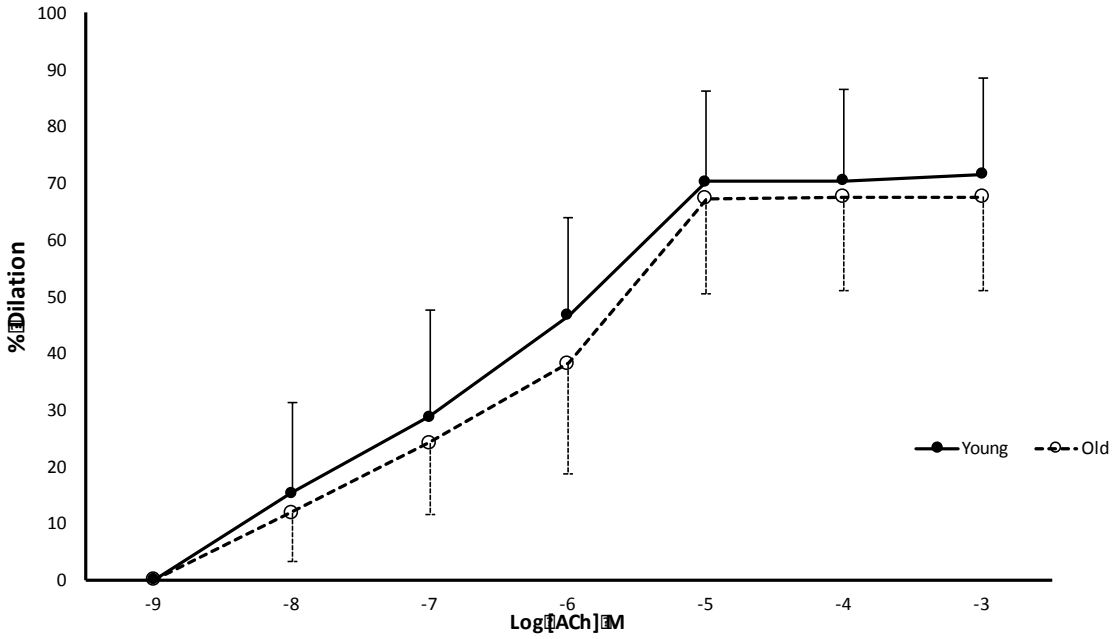

FIGURE 9. EFFECTS OF AGEING ON THE DILATOR RESPONSE TO ACH.

ACh (10^{-9}-10^{-3} M)-induced concentration-dependent dilation in isolated pressurized femoral arteries from 4-(young; n = 11) and 26-month old mice (old; n = 6). We observed no significant effect of ageing on ACh-induced dilation between groups. Data are means ± SEM.

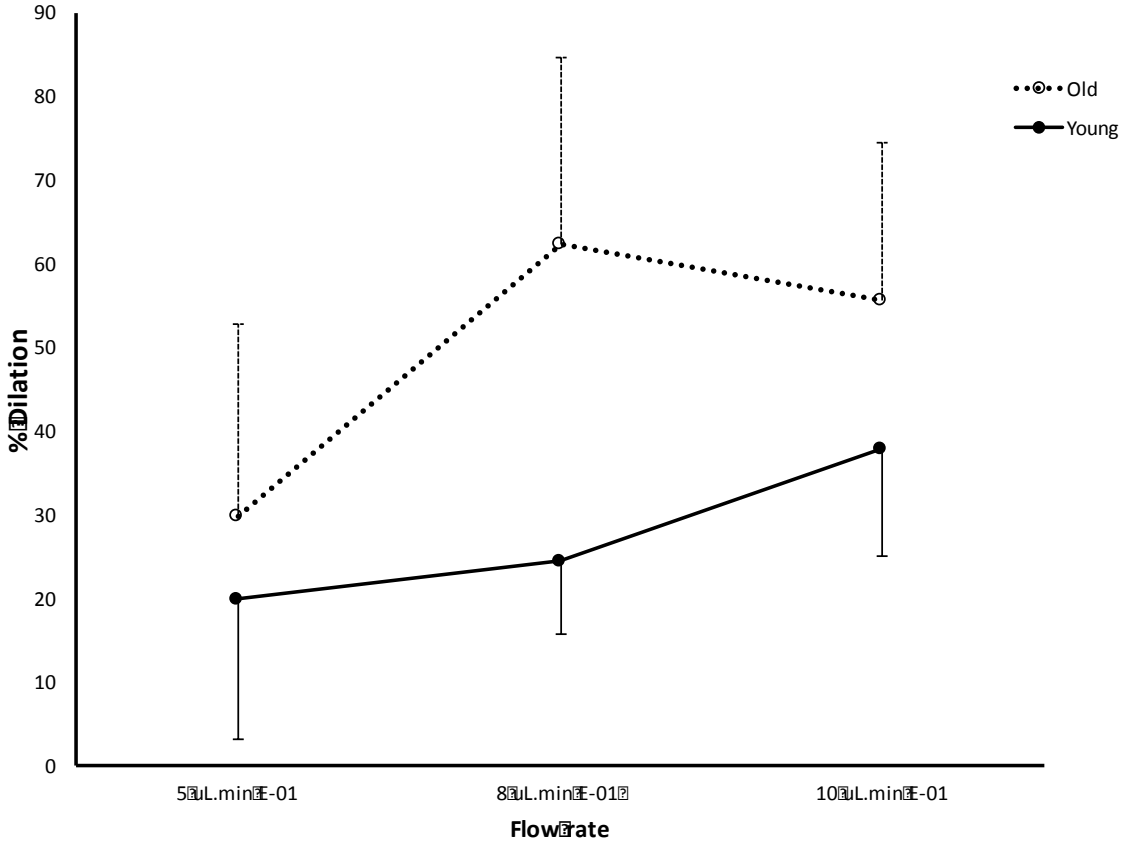

FIGURE 10. EFFECTS OF AGEING ON THE DILATOR RESPONSE TO INTRALUMINAL FLOW.

Intraluminal flow (5-10 μL·min^{-1}) induced dilation in isolated pressurized femoral arteries from 4-(young; *n* = 11) and 26-month old mice (old; *n* = 6). However, we observed no significant effect of ageing on ACh-induced dilation. Data are means ± SEM.

TABLE 1. SHEAR STRESS (DYN·CM^2) IN SEGMENTS OF THE ISOLATED PRESSURIZED MOUSE FEMORAL ARTERY IN RESPONSE TO INTRALUMINAL FLOW.

Values are means ± SEM.

	FLOW RATE		
EXPERIMENTAL GROUP	**5 μL·min^{-1}**	**8 μL·min^{-1}**	**10 μL·min^{-1}**
Young PSS	66 ± 48	10 ± 7	80 ± 60
Old PSS	117 ± 148	107 ± 148	36 ± 28
Young LNNA	18 ± 5	27 ± 6	32 ± 7
Old LNNA	14 ± 12	22 ± 18	28 ± 23
Young Apamin + Tram34	5 ± 4	7 ± 4	8 ± 6
Old Apamin + Tram34	14 ± 10	22 ± 16	28 ± 23
Young Indomethacin	14 ± 8	24 ± 14	30 ± 18
Old Indomethacin	12 ± 5	19 ± 8	23 ± 10

Characterization of dilator responses

ACh-induced dilation

Figures 11 and 12 show the dilator responses of the mouse femoral artery to ACh in the presence of LNNA, apamin + Tram 34, or indomethacin. The ANOVA revealed no significant effects of treatment, age or the interaction between the two for ACh-induced dilation.

Maximal flow-mediated dilation

Intraluminal flow caused increases in lumen diameter of the isolated pressurized mouse femoral artery during the first minute of introduction. We observed significant effects of flow and the interaction between flow and inhibitor. In the young, addition of LNNA or apamin + Tram 34 significantly reduced maximal FMD (Figure 13; $p<0.05$). We observed no significant effects of indomethacin (Figure 13). On the other hand, all treatments significantly reduced maximal FMD in the old (Figures 14; $p<0.05$). Of note are the effects of LNNA and indomethacin, which abolished any increase in

diameter in response to flow (Figure 14). At no point did intraluminal flow cause constriction of the vessels.

Sustained flow-mediated dilation

The increase in lumen diameter of the isolated pressurized mouse femoral artery in response to intraluminal flow was stable for approximately 2-3 minutes. In general, arteries from both young and old mice returned to pre-constricted levels prior cessation of flow. We found a significant effect for the difference between the dilation upon the introduction of flow and the sustained dilation prior cessation of flow ($p<0.05$), inhibitors ($p<0.05$), and the interaction between the two ($p<0.05$). The latter implies that the effect of inhibitors differs between time points but not between age groups. To assess the meaning of this, we calculated the ratio of the diameter at initial maximal dilation to the diameter prior cessation of flow during a given condition and found that such ratio was larger in PSS than LNNA ($p<0.05$), apamin + Tram 34 ($p<0.05$) and indomethacin ($p<0.05$). This means that the degree of dilation was less sustained in PSS than in the other conditions. Phrased differently, auto-regulation, the capacity of the vessel to adjust lumen diameter back to its pre-constricted level in the constant presence of flow, was significantly compromised upon addition of inhibitors of endothelial factors in both young and old (Figures 15-

17). Figure 18 shows a representative trace of the transient dilator response to intraluminal flow in young and old mice.

Taken together, the above findings indicate that in the isolated pressurized mouse femoral artery, ageing (from 4 to 26 months) has no significant effect on ACh (10^{-9}-10^{-3} M)-induced and flow (5-10 $\mu L \cdot min^{-1}$)-mediated dilation. However, within age groups there are differences in the contribution of endothelial factors to maximal FMD. Addition of LNNA or apamin + Tram 34 significantly reduced maximal FMD in both groups. However, addition of indomethacin had no effects on maximal FMD in the young whereas it abolished it in the old. Further, that in addition to maximal FMD there is a temporal component to the dilator response to intraluminal flow; sustained FMD. In response to intraluminal flow, the isolated pressurized mouse femoral artery increases in diameter, which as represented in Figure 10 is maintained stable for approximately 2-3 minutes. The artery auto-regulates its diameter back to pre-constricted levels regardless of age. The inhibition of endothelial factors significantly compromises such auto-regulation.

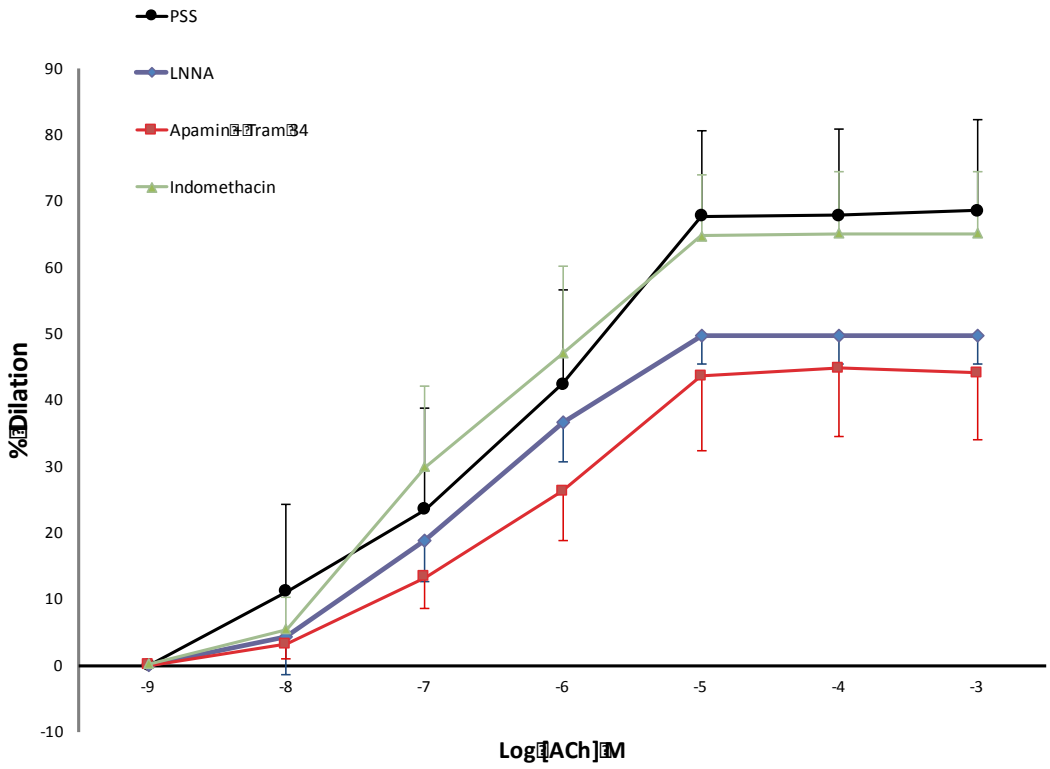

FIGURE 11. CHARACTERIZATION OF THE DILATOR RESPONSE TO ACH IN THE YOUNG.

Incubation of isolated pressurized femoral arteries from 4-month old mice with of LNNA (100 uM; *n* = 6), indomethacin (10 uM; *n* = 6), or the combination of apamin (100 nM) and Tram 34 (1 uM) (*n* = 6) had no significant effects of ACh-induced dilation. Data are means ± SEM.

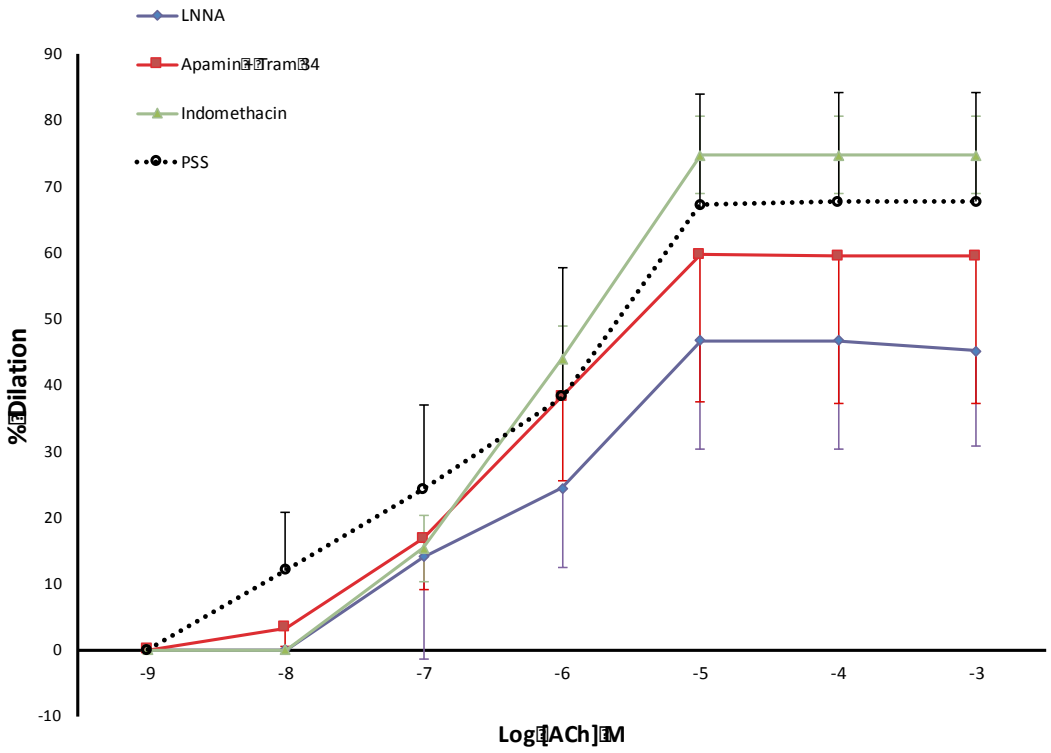

FIGURE 12. CHARACTERIZATION OF THE DILATOR RESPONSE TO ACH IN THE OLD.

Incubation of isolated pressurized femoral arteries from 26-month old mice (old) with of LNNA (100 uM; *n* = 6), indomethacin (10 uM; *n* = 6), or the combination of apamin (100 nM) and Tram 34 (1 uM) (*n* = 8) had no significant effects of ACh-induced dilation. Data are means ± SEM.

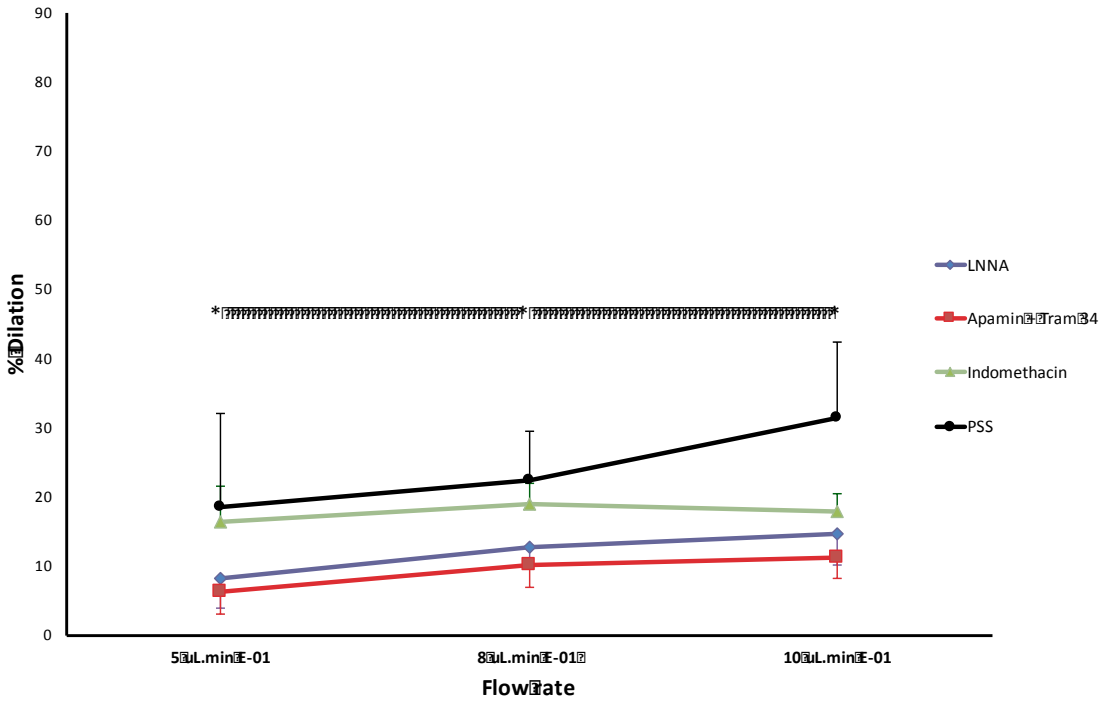

FIGURE 13. CHARACTERIZATION OF THE DILATOR RESPONSE TO INTRALUMINAL FLOW IN THE YOUNG.

Incubation of isolated pressurized femoral arteries from 4-month old mice with LNNA (100 uM; *n* = 6), or the combination of apamin (100 nM) and Tram 34 (1 uM) (*n* = 6) had significant effects on FMD when compared to controls (PSS; $p<0.05$). Addition of indomethacin (10 uM; *n* = 6) had no significant effect on FMD. Data are means ± SEM.

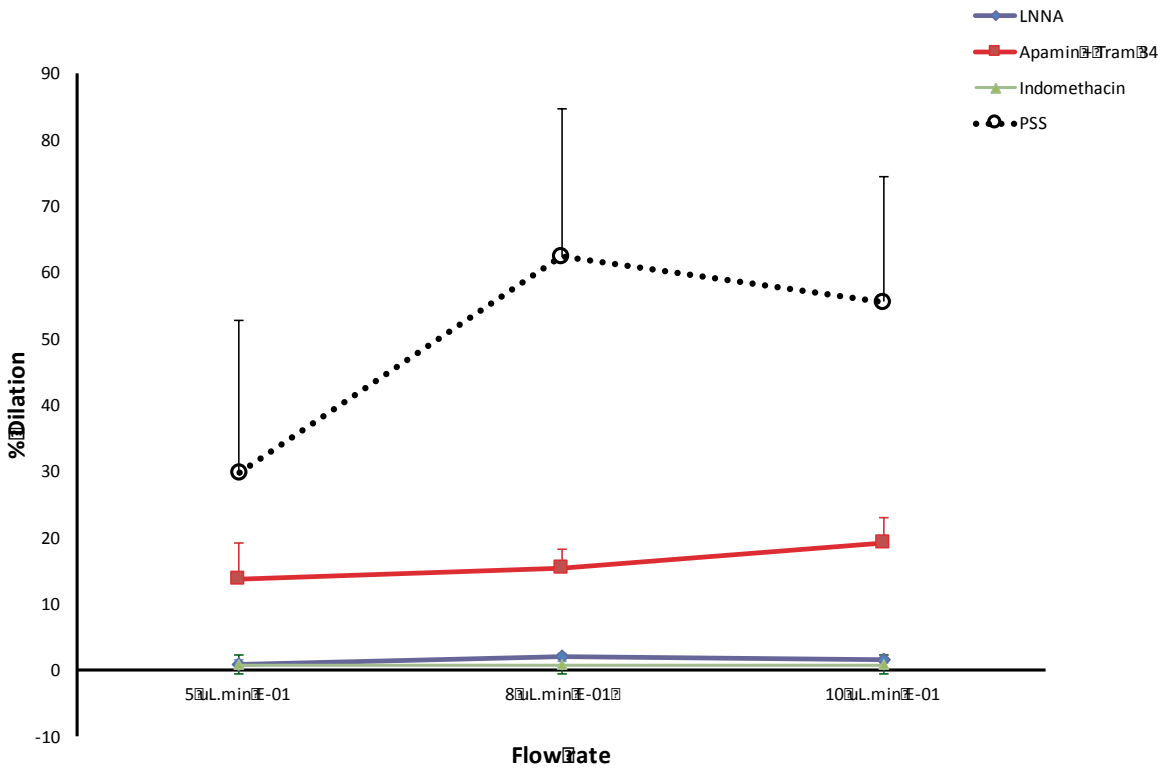

FIGURE 14. CHARACTERIZATION OF THE DILATOR RESPONSE TO INTRALUMINAL FLOW IN THE OLD.

Incubation of isolated pressurized femoral arteries from 26-month old mice with LNNA (100 uM; *n* = 6), indomethacin (10 uM; *n* = 6), or the combination of apamin (100 nM) and Tram 34 (1 uM) (*n* = 8) had significant effects on FMD. The ANOVA revealed significant differences in each inhibitor of endothelial factors on FMD when compared to controls (PSS) ($p<0.05$). Data are means ± SEM.

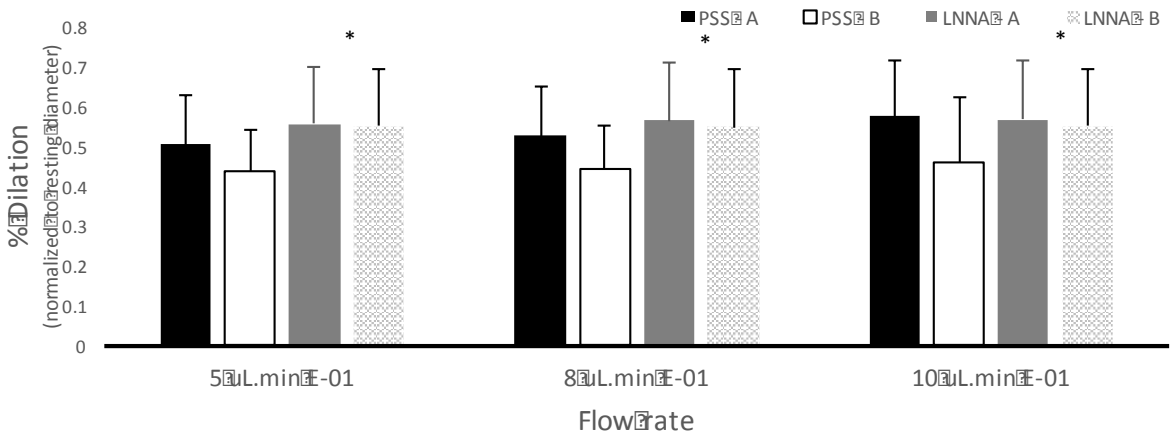

FIGURE 15. EFFECTS OF LNNA ON THE SUSTAINED FMD OF ISOLATED PRESSURIZED MOUSE FEMORAL ARTERIES.

Incubation of isolated pressurized femoral arteries from 4-(young) and 26-month old mice (old) with LNNA (100 uM; *n* = 6 per group) significantly reduced the capacity of the vessel to auto-regulate tone in the presence of intraluminal flow (5-10 $\mu L \cdot min^{-1}$) in both groups. The ratio between the initial maximal diameter upon introduction of flow to the sustained diameter prior cessation of flow was significantly lower in the presence of LNNA ($p<0.05$). A - maximal diameter upon introduction of flow. B – sustained diameter prior cessation of flow. * The ratio between the initial diameter upon introduction of flow to the sustained diameter prior cessation of flow under LNNA is different from PSS at $p<0.05$. Data are means ± SEM.

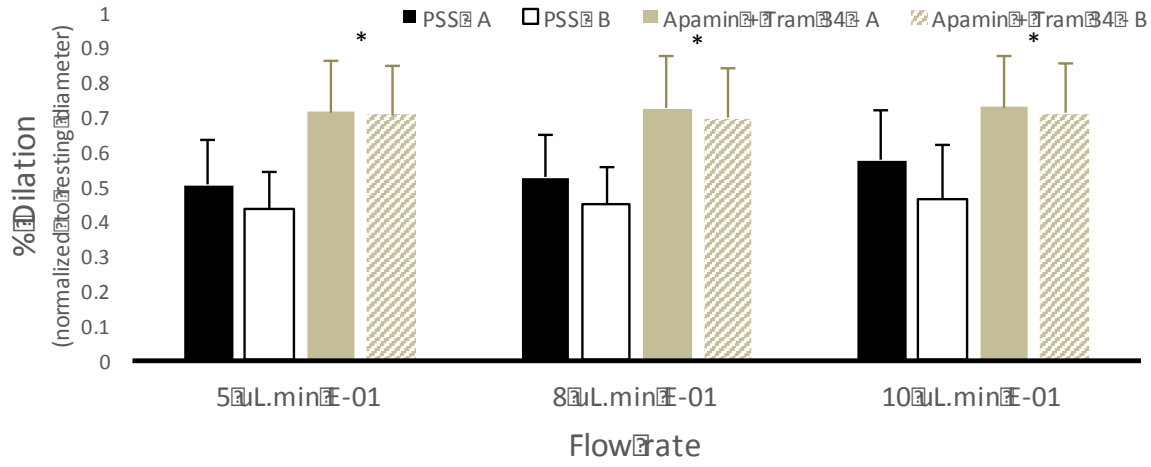

FIGURE 16. EFFECTS OF APAMIN + TRAM 34 ON THE SUSTAINED FMD OF ISOLATED PRESSURIZED MOUSE FEMORAL ARTERIES.

Incubation of isolated pressurized femoral arteries from 4-(young) and 26-month old mice (old) with apamin (100 nM) and Tram 34 (1 uM) (*n* = 6 and 8 for young and old groups, respectively) significantly reduced the capacity of the vessel to auto-regulate tone in the presence of intraluminal flow (5-10 $\mu L \cdot min^{-1}$) in both groups. The ratio between the maximal diameter upon introduction of flow to the sustained diameter prior cessation of flow was significantly lower in the presence of apamin and Tram 34 ($p<0.05$). A - maximal diameter upon introduction of flow. B – sustained diameter prior cessation of flow. * The ratio between the initial diameter upon introduction of flow to the sustained diameter prior cessation of flow under apamin + Tram 34 is different from PSS at $p<0.05$. Data are means ± SEM.

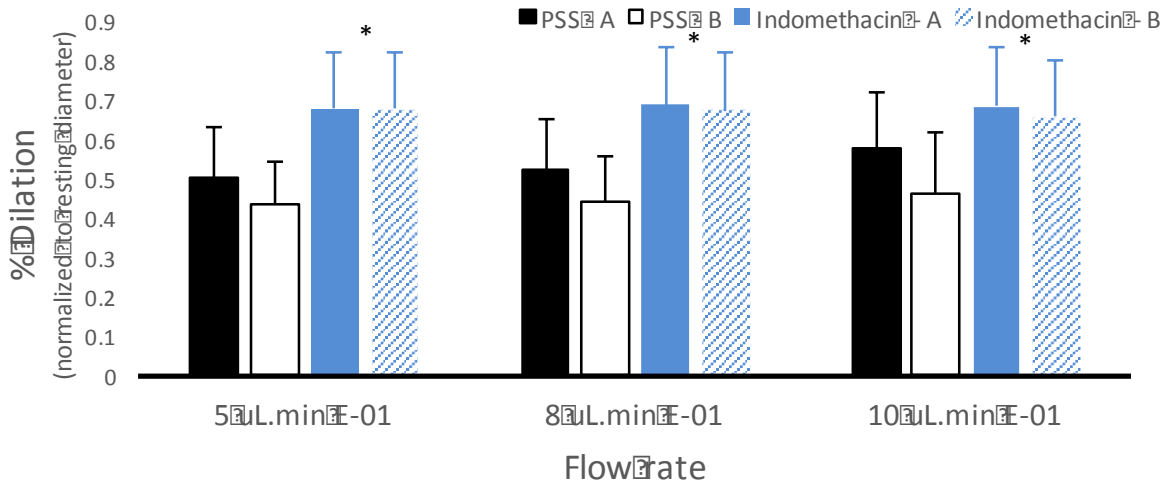

FIGURE 17. EFFECTS OF INDOMETHACIN ON THE SUSTAINED FMD OF ISOLATED PRESSURIZED MOUSE FEMORAL ARTERIES.

Incubation of isolated pressurized femoral arteries from 4-(young) and 26-month old mice (old) with indomethacin (10 uM; *n* = 6 per group) significantly reduced the capacity of the vessel to auto-regulate tone in the presence of intraluminal flow (5-10 $\mu L \cdot min^{-1}$) in both groups. The ratio between the maximal diameter upon introduction of flow to the sustained diameter prior cessation of flow was significantly lower in the presence of indomethacin ($p<0.05$). A- maximal diameter upon introduction of flow. B – sustained diameter prior cessation of flow. * The ratio between the initial diameter upon introduction of flow to the sustained diameter prior cessation of flow under indomethacin is different from PSS at $p<0.05$. Data are means ± SEM.

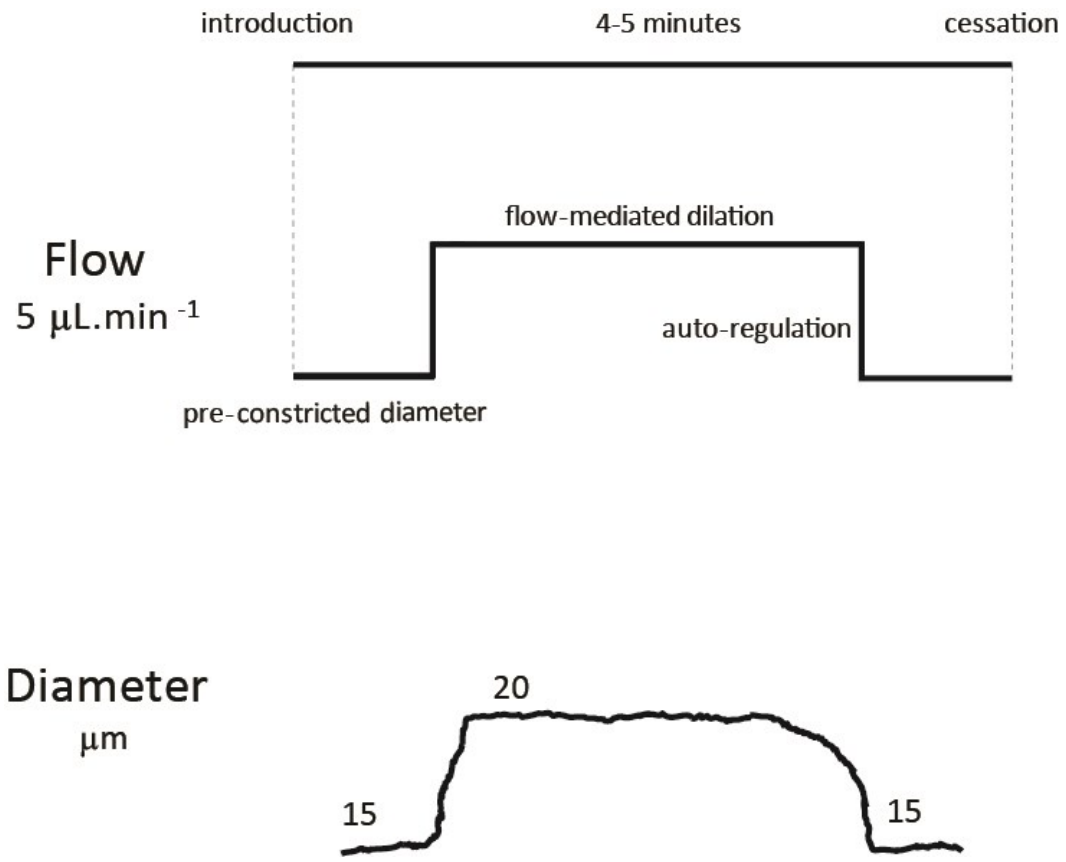

FIGURE 18. TRANSIENT DILATOR RESPONSE TO INTRALUMINAL FLOW.

Representative trace of the transient dilator response to intraluminal flow (5 µL·min^{-1}) of the Phe (10^{-5} M)- pre-constricted isolated pressurized femoral artery from a 26-month old mouse. In general, responses to intraluminal flow in both young and old mice were maximal during the first minute, followed by a plateau phase that typically lasted 2-3 minutes and a rapid return to pre-constriction levels prior cessation of flow. The upper panel shows the introduction and cessation to flow. The bottom panel shows the actual changes in diameter in response to intraluminal flow.

Discussion

We investigated the influence of ageing on endothelium-dependent dilation of the isolated pressurized mouse femoral artery. We determined the NO-dependent and –independent components of vessel dilation in response to ACh and intraluminal flow. The main findings of this study are that the mouse femoral artery does not show altered responses to either Phe, ACh or intraluminal flow with age. Secondly, that with ageing there is a shift in the role that PGI_2 plays in maximal FMD. Whereas addition of indomethacin had no effects of maximal FMD in the young, it significantly reduced it in the old. Finally, that there is an auto-regulatory component to FMD. It is preserved with age and significantly reduced upon the addition of inhibitors of endothelial factors.

Endothelial function and ageing

Contrary to our hypothesis, we observed no attenuation in dilation (a marker of endothelial function) to either ACh or intraluminal flow in the femoral artery of aged mice. Some studies on vascular function indicate that ageing blunts NO-dependent relaxation. Hence, compromising endothelium-mediated dilation of the aorta (Loo et al., 2000), mesenteric artery (Dal-Ros et al., 2012) and coronary arterioles (Csiszar et al., 2002) of rats. However, it has been demonstrated that the effects of ageing on the

vasculature supplying the skeletal muscle vary according to branch order and the nature of the dilator stimulus (Sinkler and Segal, 2014). Thus far, the studies on the mouse femoral artery are limited. Our findings are in agreement with some (Wang et al., 2000; Bearden et al., 2004; Didion et al., 2006; Baron et al., 2014; Sinkler and Segal, 2014), but not all (Schuler et al., 2014; Jelinic et al., 2015; Rammos et al., 2015) of the reports that have investigated the effects of ageing on dilation of conduit arteries or vascular beds supplying the skeletal muscle. In their report, Sinkler and Segal (2014) observed that the maximal internal diameter induced by ACh of the feeding artery and first-order arterioles of the gluteus maximus from 24-month mice was not different from their 4-month counterparts. In fact, second- and third-order arterioles from the old animals showed enhanced dilation to doses of ACh 10^{-7} M and higher. Further, ACh-induced dilation was proportional to that evoked by sodium nitroprusside. Together, this suggests that the endothelium was fully able to drive vascular smooth muscle relaxation without any age-related complications (Sinkler and Segal, 2014). These findings are in line with those reported by Bearden et al (2004), who demonstrated a conserved architecture of arteriolar networks in the gluteus maximus muscle of young (3 months), adult (12 months) and old (20 months) mice. This was accompanied by similar dilator and constrictor responses to ACh and Phe, respectively (Bearden et al., 2004). In line with this, we did not observe any age-related differences in internal diameter of the isolated pressurized femoral artery or in the vascular responses to agonists (ACh and Phe). On the other hand, isolated aortic rings from 20-month old mice showed decreased dilation to doses of ACh 10^{-7} M and higher when compared to 6-month old controls (Rammos et al., 2015). The

discrepancy in the findings reported thus far in the literature is most likely related to the vascular bed investigated. Previous studies have suggested that the functional differences in the vascular endothelium and smooth muscle through the vascular tree can be explained by a number of factors, such as the expression of agonist receptors, structural proteins and the regulation of signal transduction pathways critical to vessel dilation (Hill et al., 2001; Newcomer et al., 2004). In experimental animals, it has been shown that compromised endothelial function is the consequence of sustained elevations in sympathetic stimulus and the associated hypertrophy of smooth muscle (Pauletto et al., 1991; Cipolletta et al., 2010; Gradinaru et al., 2015). In support of this hypothesis, Sinkler and Segal (2014) demonstrated that mice that did not develop age-related endothelial dysfunction in the arterioles of the gluteus maximus showed a reduced constriction to Phe due to lower activation of α-adrenoreceptors (Sinkler and Segal, 2014). Of note, in our study we observed no statistical difference in the response to Phe 10^{-5} M between 4- and 26-month old mice.

ACh-induced dilation

It is well known that there is a cross-talk between the different endothelium-derived factors modulating dilation (Azzawi and Austin, 2007) and that the role of such factors can be influenced by ageing or disease (Ueda et al., 2005). The ACh-induced and flow-mediated endothelium-dependent dilation of arteries depend on the

interplay of NO, EDHFs and PGI_2. Therefore, we characterized the dilator response of the mouse femoral artery to ACh and intraluminal flow by inhibiting the production of such molecules. In our study, we found unchanged levels of ACh-induced dilation of the isolated pressurized femoral artery with ageing, although not statistically significant, the increase in diameter in response to ACh was reduced in both young and old upon treatment with LNNA or the combination of apamin + Tram 34. Treatment with indomethacin had little impact on maximal diameter. It is worth mentioning at this point that the lack of statistical significance when evaluating the contribution of NO, EDHFs and PGI_2 to vessel dilation, as observed in our ACh dose responses, does not necessarily exclude biological relevance. The contribution of each molecule to smooth muscle relaxation and consequently, to vasodilation might be proportional, which would negate mathematical differences. However, it bears considerable physiological meaning. The lack of statistical differences amongst the ACh dose responses of the isolated pressurized mouse femoral artery upon treatment with inhibitors suggests redundancy amongst the pathways contributing to vasodilation. Evidence of the existence of conserved and compensatory pathways regulating endothelium-dependent vasodilation in other vascular beds has been presented in the soleus feed arteries of 4- and 24-month old Fischer 344 rats, in which addition of LNNA or indomethacin had no effect in lumen diameter in response to ACh 10^{-9}-10^{-4} M when compared to controls (Woodman et al., 2003). Similar results have been observed in the cutaneous microvessels of 25-month old C57BL/6 mice, in which treatment with LNNA and indomethacin had no effect in ACh-induced dilation (Gaubert et al., 2007). The existence of compensatory pathways for vasodilation is

most likely beneficial for cardiovascular health to the extent that they might compensate for the loss of specific vasodilators caused by disease (Ballard et al., 2014). On the other hand, we acknowledge that the large variation in the ACh-induced dilator responses presented in this study is a limitation and might have played an important role in the lack of statistical significance in lumen diameter upon treatment with inhibitors. However, for some groups we increased the number of vessels up to 11 in order to reduce variation with little, if any effect on mean values and standard errors.

Maximal flow-mediated dilation

In addition to ACh-induced dilation, endothelial function can be assessed by means of FMD. The dilation of arteries in response to increases in wall shear stress is in fact more physiologically relevant since it simulates more closely *in-vivo* behaviour (Shahid and Buys, 2013). Similar to ACh-induced dilation, we found a preserved maximal FMD with age. In response to intraluminal flow rates of 5 and 8 $\mu L \cdot min^{-1}$, we observed higher mean shear stress values in the aged arteries. Even though variation is large, this could partially explain the preserved levels of maximal FMD with age. On the other hand, unlike ACh-induced dilation, we observed significant differences in the contribution of endothelial factors to maximal FMD with ageing. Of interest is the fact that initial maximal FMD seems to rely on NO and EDHFs in the young with little

contribution of PGI_2. This is in stark contrast with the aged arteries in which addition of indomethacin led to negligible levels of dilation. Our findings are partially in agreement with a previous report that studied age-related changes in FMD of the mouse femoral artery (Schuler et al., 2014). Using high-frequency ultrasound, Schuler and colleagues demonstrated that FMD of the mouse femoral artery is mostly NO-dependent. Also, that eicosanoids play little, if any, role in dilation in 12-week old male mice. Finally, that there is an age-related decrease in the NO-dependent component of FMD. In light of such decrease, and contrary to our study, the authors found a significant loss of endothelial function in 24-month old mice (Schuler et al., 2014). Similarly, FMD of the femoral artery in 12-week old rats is reported to be completely NO-dependent and significantly higher than in 24-week old animals in which NO accounts only for half of maximal dilation. Since the remainder of maximal FMD was blocked with apamin and charybdotoxin (a Ca^{2+}-activated K^+ channel blocker) the authors suggested a more predominant age-related contribution of EDHFs (Heiss et al., 2008). Finally, in the soleus feed arteries of Fischer 344 rats, inhibition of NO or PGI_2 with LNNA or indomethacin, respectively, significantly reduced FMD in 4-month, but not in 24-month animals (Woodman et al., 2003). It has been proposed that such changes reflect back-up mechanisms that compensate for the loss of NO bioavailability, or the release of other endothelial factors, particularly during the late stages of life or in pathological states (Bauersachs et al., 1996; Sofola et al., 2002; Puzserova et al., 2014). Our findings confirm previous published studies that indicate a shift in the predominance that endothelial factors play in vessel dilation of isolated pressurized arteries in response to intraluminal flow.

Sustained flow-mediated dilation

The study of the temporal characteristics of FMD allows for a comprehensive understanding of how arteries regulate tone. Here, we have demonstrated for the first time that in the presence of inhibitors, the diameter of the isolated pressurized mouse femoral artery in response to intraluminal flow does not decrease over time. In our study, the dilation responses to intraluminal flow were maximal during the first minute, followed by a plateau phase that typically lasted <2-3 minutes. Overall, such responses were preserved with age. These data clearly demonstrate that there is a further level of complexity, i.e., sustained dilation, that needs to be considered when investigating the effects of ageing on FMD. To our knowledge, this is the first study to characterize the dilator responses to intraluminal flow of the isolated pressurized mouse femoral artery. Hence, we are unable to compare our findings. Thus far, only a handful of studies have investigated the sustained responses to intraluminal flow *ex-vivo* in other vascular beds. In rat mesenteric arteries, Liu et al., (2004) demonstrated transient responses to intraluminal flow (shear stress 3.5–10.7 dyn·cm^2), which became more stable upon addition of ATP 1 μM. Further, in first-order arterioles of the rat soleus and gastrocnemius muscles, Shipley et al., (2005), reported that incubation with LNNA inhibited the sustained (20 minutes) FMD but not the initial maximal dilation (first two minutes). Indomethacin had no effects on either initial maximal dilation or sustained dilation in these vessels. In arterioles of the rat gracilis muscle, Sun et al., (2001) showed an oscillatory FMD that quickly decreased after flow

rates of 5 μL·min^{-1} but increased again in response to flow rates >15 μL·min^{-1} (shear stress 50 – 180 dyn·cm^2) remaining stable up to five minutes. Opposite to this, Azzawi and Austin (2007) reported that in rat coronary arteries, the dilator responses to flow become more transient in nature with increasing flow. Whereas flow rates of 5-25 μL·min^{-1} provoked a maximal dilation that is maintained up to four minutes, the dilation induced by higher flow rates is sustained for less than one minute. Contrary to our study, both LNNA and indomethacin led to a more transient response to flow.

It has been well established that the chemical factors mediating vasodilation might not be the same factors that sustain it (Joyner and Casey, 2015). In conduit arteries supplying the skeletal muscle, such as the femoral artery, sustained dilation would be critical during prolonged physical effort. Skeletal muscle contraction compresses blood vessels reducing blood flow. On the other hand, the changes in pressure caused by relaxation allows blood vessel to dilate and blood flow to increase exponentially (Tschakovsky and Sheriff, 2004). Hence, the skeletal muscle pump is a critical factor for the rapid increase in muscle blood flow observed during moderate physical effort. However, the high mechanical forces of prolonged and strenuous effort might offset the flow-promoting effects of muscle contraction (Lutjemeier et al., 2005). Clearly, the increase in endothelium-mediated blood flow during prolonged physical effort is decisive for an adequate delivery of nutrients and oxygen to the active muscle. Failing to meet the demands of the muscle would lead to a reduced physical capacity and a diminished potential to grow and adapt. Suggestive evidence in this sense already

exists. Recently, it has been reported that chronic exercise preferentially enhances the sustained endothelium-dependent bradykinin-induced relaxation of small coronary arteries in pigs (Heaps et al., 2014). According to the authors, in this vascular bed initial maximal dilation is mediated by NO and prostanoid-independent mechanisms, most likely EDHFs whereas sustained dilation is predominantly mediated by NO (Heaps et al., 2014). However, our assumptions are somewhat speculative and need to be examined in more detail. Age-related shifts in the contribution that NO, EDHFs and PGI_2 play in vasodilation need to be taken into account when designing and prescribing pharmaceuticals aiming to improve endothelial function in cardiovascular disease. Whereas prostacyclin analogs such as epoprostenol or treprostinil might be more appropriate in the elderly than in young subjects, NO donors might be suitable for both age groups. Further, the transient dilator response to flow is mechanistically of great interest. Considering the wide-spread use of COX inhibitors for their pain-relieving and anti-inflammatory properties, the role of PGI_2 on sustained FMD in humans, particularly in the elderly, warrants investigation (Azzawi and Austin, 2007).

Conclusions

Taken together, our findings indicate that the isolated pressurized mouse femoral artery does not develop compromised endothelial function from 4 to 26-months of age. Constrictor response to KPSS, Phe and dilator responses to ACh and intraluminal

flow are of similar magnitude between the femoral arteries from young and old mice. The mechanisms that govern dilation differ from the type of stimulus (agonists *vs*. flow). The qualitative shift in the contribution to dilation of different vasoactive molecules is not associated with blunted levels of maximal dilation induced by either ACh or intraluminal flow. FMD, the more physiologically relevant measure of endothelium-dependent dilation, exhibits temporal characteristics that extend beyond maximal dilation. Different from initial maximal dilation, sustained dilation seems to be mediated by NO, EDHFs and PGI_2 regardless of age. The understanding of the contribution of vasoactive substances towards vasodilation with ageing is essential for the development of successful pharmacological and/or nutraceutical interventions that aim to improve cardiovascular health.

Chapter 3

Differential effects of resveratrol on the dilator responses to acetylcholine and intraluminal flow of the isolated mouse femoral artery

Abstract

A large body of evidence supports the consumption of fruit and vegetables to prevent cardiovascular disease. Here, we investigated the effects of *trans*-resveratrol (RV; 45 μM), a polyphenol present in red grapes, berries and peanuts on acetylcholine (ACh)-induced and flow-mediated dilation (FMD) of the isolated pressurized femoral artery of young and old mice. RV significantly enhanced ACh (10^{-5} – 10^{-3} M)-induced dilation in young and old mice ($p<0.05$). In contrast, it completely abolished FMD (5-10 $\mu L \cdot min^{-1}$) ($p<0.05$). Further, RV significantly reduced the capacity of the isolated pressurized mouse femoral artery to regulate its diameter in the constant presence of flow ($p<0.05$). Following RV incubation, the production of NO by cultured endothelial cells in response to ACh (10^{-5} M) or intraluminal flow (5-10 $\mu L \cdot min^{-1}$) for 10 minutes was significantly reduced ($p<0.05$). However, ACh significantly increased NO production when cells were challenged for one hour ($p<0.05$). The effects of RV on ACh-induced and FMD seem to be NO-independent and dependent, respectively. The effects of RV on FMD indicate that RV might compromise flow-mediated endothelial mechanotransduction. Our findings raise important questions regarding the insight gained from *ex-vivo* models. Investigating vasodilation in the absence of flow might not provide a comprehensive understanding of the effects of RV on endothelium-dependent dilation.

Keywords: Flow-mediated dilation; Ageing; Endothelial function; Pressure Myography; Nitric Oxide.

Introduction

A large body of evidence supports the consumption of fruit and vegetables to prevent cardiovascular disease (CVD) (Brat et al., 2006; Nicholson et al., 2010). The protective effects of these foods are, amongst others, attributed to the presence of polyphenols, chemical compounds with anti-inflammatory and anti-oxidant properties (Pandey and Rizvi, 2009). Amongst the polyphenols that have received most attention in the last decade is RV (Joao Tomé-Carneiro et al., 2012; João Tomé-Carneiro et al., 2012, 2013). Found mostly in berries, peanuts and the skin of red grapes, RV has been shown to inhibit LDL oxidation (Brito et al., 2002), and to prevent platelet aggregation via the preferential inhibition of COX-1 *in-vitro* (Kutil et al., 2014). Further, accumulating evidence *ex-vivo* advocates the vasorelaxant effects of RV. In rat aortic and mesenteric rings, RV has been shown to promote dilation of pre-constricted vessels (Chen and Pace-Asciak, 1996; Orallo et al., 2002), improve endothelium-dependent ACh-induced dilation (da Luz et al., 2012; Gocmez et al., 2016) and flow-mediated outward remodelling after ligation (Petit et al., 2016). Recently, it was demonstrated that RV improves both endothelium-dependent and -independent dilation of isolated bovine retinal arteries (Vanden Daele et al., 2016). In addition, RV restored endothelium-dependent ACh-induced dilation in the isolated mouse aorta impaired by high glucose (Hu and Liu, 2016). These responses are accompanied by reductions in BP in spontaneously hypertensive rats (Li et al., 2016) and are thought to be primarily, though not completely, mediated by increases in the expression and

activity of eNOS, and the bioavailability of NO (Wallerath et al., 2002; Nagaoka et al., 2007; Breen et al., 2012; Li et al., 2016) .

In-vivo, chronic supplementation with RV is reported to improve FMD of the brachial artery of elderly patients with metabolic syndrome and CAD (Fujitaka et al., 2011; Magyar et al., 2012). In the latter study, however, the improvements in FMD were accompanied by an approximate 50% increase in C-reactive protein and TNF-α (Magyar et al., 2012), markers of inflammation associated with endothelial dysfunction (Picchi et al., 2006; Hein et al., 2009). By contrast, recent studies suggest that RV has no benefit in a broad range of cardiovascular parameters and even negates the effects of physical exercise. For instance, RV blunted the exercise-induced increase in the capillary-to-fibre ratio of the vastus lateralis muscle of aged men after eight weeks of physical exercise (Gliemann et al., 2014). Further, RV reduced the increase in PGI_2 whilst promoted the increase in muscle thromboxane synthase. RV also blunted the exercise-induced effects on BP, the circulating levels of LDL, cholesterol, triglycerides (Gliemann et al., 2013), protein carbonylation and TNF-α (Olesen et al., 2014). Although still quite small in number, these findings challenge the translational potential of RV to improve endothelial function that has been gathered from *in-vitro* and *ex-vivo* experimentation and few human studies. The discrepancy amongst these lines of experimental evidence might be attributed to expected issues such as the dose of RV used, the vascular bed investigated and the species in which the studies were conducted (Gescher and Steward, 2003; Joao Tomé-Carneiro et al.,

2013). However, particular attention should be paid to RV metabolism. It is well known that polyphenols are extensively metabolized by intestinal and hepatic enzymes (Manach et al., 2004). Hence, their bioavailability in blood varies significantly, particularly when consumed orally. In humans, the bioavailability of RV is very low despite high levels of absorption (>70%) (Walle et al., 2004). A single dose of RV 25 mg results in peak levels of RV metabolites of 2 μM and only trace amounts of non-metabolized RV (Walle et al., 2004). Therefore, it is likely that *in-vivo* the putative positive effects of RV consumption on FMD are mediated by secondary metabolites (Walle et al., 2004). This line of enquiry is important because most of the studies published thus far *in-vitro* and *ex-vivo* have investigated the effects of total *trans*-RV, but not its metabolites. In order to compare published findings, it is necessary to identify the exact molecule to which the target cell/tissue is exposed. Recently, it was demonstrated *in-vitro* that *trans*-RV, but not any of the metabolites expected to be found in blood after its oral consumption, increases the activity and gene expression of eNOS in EC and reduces intracellular ROS levels (Ladurner et al., 2014). Considering that by default *in-vitro* and *ex-vivo* experimentation bypass digestion, absorption and form of delivery to target tissues, the inconsistency with the findings gathered *in-vivo* should have been better anticipated. Elaborating on these issues becomes critical to validate the potential of RV as a nutraceutical strategy in the prevention and treatment of CVD. Surprisingly, this is something that has received very little attention to date.

Here, we determined the degree of RV purity and stability during functional studies with pressure myography of isolated, pressurized mouse femoral arteries. More importantly, we investigated *ex-vivo* the effects of RV as a bioactive compound on dilation of the isolated pressurized mouse femoral artery to increasing doses of ACh and intraluminal flow in young and old mice. To compare the findings from our functional studies with those gathered from studies *in-vitro*, we tested the effects of RV on the production of NO in response to ACh and laminar flow in cultured EC. Consequently, the present study constitutes an attempt to shed light on the effects of RV on vessel dilation. The particular aims of this study were to 1) determine the stability of RV during experimental settings of pressure myography; 2) determine to what extent ACh-induced and FMD of the femoral artery from young (4 months), and aged (26 months) mice can be improved with RV, *ex-vivo*; and 3) determine the effects of RV on the production of NO by cultured EC in response to ACh and laminar flow. We hypothesised that 1) RV would improve ACh-induced and FMD of the isolated pressurized femoral artery in young and old mice and 2) such enhancement is dilation would be associated with an increased production of NO.

Methods

High-performance liquid chromatography

All solvents (water, ethanol and acetonitrile) were of HPLC grade or higher, and obtained from Fisher Scientific (Loughborough, UK). Sodium azide, EDTA, ascorbic acid and RV reference standard were obtained from Sigma (Dorset, UK). Taxifolin was obtained from Extrasynthese (Vichy, France). All reference standards were of HPLC grade (>95%).

Preparation of RV test samples

RV test material was supplied in hard-shelled capsules by 21st Century Alternatives (UK) that contained 330 mg, and was stated to contain RV at >98% purity. A total of three capsules were weighed, and the powdered contents removed. From each powdered capsule, an aliquot of approximately one milligram was weighed into a 2-mL amber centrifuge tube (Eppendorf, UK), in duplicate. To each aliquot one millilitre of extraction solvent was added, comprising aqueous ethanol (50% v/v), ascorbic acid (0.1% w/v), and taxifolin as internal standard (100 mg·mL). The mixture was vortexed for 30 seconds, placed in an ultrasonic water bath for 20 minutes, and then

vortexed again to extract soluble materials. The tubes were then centrifuged at 13,000 rpm (10 minutes; Microcentaur, MSE, London, UK) and the supernatant removed. A second 1-mL aliquot of extraction solvent was added to the pellet, and the extraction process repeated. A 1 in 10 dilution of the first and second extract was made using extraction solvent, and all samples (four per tablet) were placed in amber vials and analysed via HPLC. Typically, >95% of target analytes were present in the first extract.

Prior to, and following incubation of the segments of the femoral artery, a storage buffer solution comprising NaH_2PO_4 (0.4 M at pH 3.6), ascorbic acid (20% w/v) and EDTA (0.1% w/v) was added (10% v/v) to aliquots of RV-enriched buffer. Following defrosting at room temperature, samples were vortexed, diluted 9 in 10 with taxifolin internal standard (1 $mg \cdot mL^{-1}$), centrifuged and the supernatants placed in amber vials and analysed via HPLC.

The analysis was conducted on a Shimadzu HPLC system comprising a Prominence delivery system (with LC-20AB binary pump, SIL-20ACHT autosampler set to 8°C, and CTO-20A column oven set to 35°C) connected to a NexeraX2 (SPD-M30A) diode array detector, scanning between 190-700 nm. A 5-µL sample aliquot was injected onto C18 150 x 2.1, 2.6 m column (ThermoFisher Scientific, Altringham, UK) fitted with a low volume "Krud catcher" pre-filter (Phenomenex UK). Samples were

separated on a binary gradient of aqueous HPLC grade acetonitrile (5 *vs*. 95% v/v, solvents A and B, respectively) plus 0.2% formic acid (v/v) running at a flow rate of 0.3 mL·min^{-1}. The gradient began at 0% solvent B, raising to 10% over five minutes, then increased linearly to 20% over 15 minutes, up to 95% in five minutes, was held for further four minutes, then decreased to 0% over one minute and was re-equilibrated over three minutes. Reponses from taxifolin and RV were read at 289 nm and 307 nm, respectively. Analytes were quantified via an 8-point standard curve plot ranging 3.75–150 mg·mL as both peak area, and peak area normalized to internal standard (Wightman et al., 2014).

Pressure myography

Femoral segments from 4- (young) and 26-month old (old) male C57BL/6 mice were dissected and mounted onto a myograph chamber as described in the previous chapter. Following mounting onto the myograph chamber and a stabilization period of 20 minutes, the viability of the femoral segments was confirmed by the presence of constrictor responses to KPSS. Segments of the femoral artery were incubated with RV 45 μM (0.1% dimethyl sulfoxide (DMSO), dissolved in PSS) for one hour. The dose of RV was chosen based on previous studies *in-vitro* showing positive effects of RV on a broad range of vascular and metabolic parameters (Bruder et al., 2001; Igura et al., 2001; Araim et al., 2002; Kao et al., 2010; Frombaum et al., 2012). All vasodilator responses to ACh and intraluminal flow were investigated as described in the

previous chapter. The characterization of the dilator responses was conducted with each inhibitor (LNNA 100 μM, apamin 100 nM + tram 34 1 μM or indomethacin 10 μM) in the presence of RV (45 μM). The experimental groups were divided as: young control (YC), young RV (YRV), old control (OC), and old RV (ORV). Preliminary data revealed that endothelium-dependent dilator responses of isolated pressurized femoral arteries are not repeatable, particularly in response to intraluminal flow. Hence, both control (YC and OC) and RV values (YRV and ORV) represent first responses. Control values refer to those presented in the previous chapter. The percentage of the dilator response to ACh and intraluminal flow was calculated as described in the previous chapter.

Cell culture

To determine the production of NO following incubation with RV, human coronary artery endothelial cells (HCAEC; PromoCell; Germany) were grown as a monolayer at 37°C under 5% CO_2 atmosphere in EC growth medium MV (PromoCell, C-22020) supplemented with 5% foetal calf serum (Invitrogen, Germany), 0.004 mL·mL of growth medium, 10 ng·mL of epidermal growth factor and 90 μg·mL of heparin. Cells from the third to sixth passage were used. Before use, the cells were washed three times in phosphate buffered saline (PBS) and then incubated with 0.1% trypsin for three minutes to detach them from the flask surface. The cells were then centrifuged at 220 *g* for three minutes and re-suspended in the growth medium for seeding.

The cells were seeded onto Nunc Thermanox coverslips (10,000 cells/coverslip; 13 mm diameter) (Thermo Fisher Scientific, USA) and allowed to proliferate for 24 hours (approximately 90% confluency). Once confluency was reached, the experiments were divided into two main parts; half of the coverslips were kept in the well plate to perform the ACh assay while the other half was transferred into a bioreactor chamber (Kirkstall, UK) attached with a flow pump system in which laminar flow was introduced as indicated below.

Determination of NO production

The cells were exposed to RV 45 μM dissolved in 1.5% DMSO (Clark et al., 2012) and growth medium for one hour to simulate the incubation period in the pressure myograph. Control cultures were treated with growth medium (1.5% DMSO) alone. After the incubation, the supernatant was collected and the cultured cells exposed to ACh 10^{-5} M for 2-10 minutes and one hour, or flow rates of 5- 10 $\mu L \cdot min^{-1}$ for 3-9 minutes. A previous study demonstrated that the bioreactor chamber can maintain cell viability and function when the flow rates are below 500 $\mu L \cdot min^{1}$, which corresponds to a wall shear stress of 10^{-5} Pa or less at the cell surface (Mazzei et al., 2010). Following exposure to ACh or laminar flow, the supernatant was collected and immediately incubated with flavin adenine dinucleotide (50 μM), reduced β-nicotinamide adenine dinucleotide phosphate (500 μM), nitrate reductase from

aspergillus species (1 u·mL^{-1}) for 15 minutes at 37°C. Then, the samples were incubated with lactate dehydrogenase (100 u·mL^{-1}) and sodium pyruvate (100 mM) for further five minutes at 37°C (Cavicchi and Whittle, 1999). The production of NO was quantified using the Griess reaction. Equal volumes of sample and Griess reagent (1% sulfanilamide in 2.5% H_3PO_4 and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride) were incubated at room temperature for 10 minutes. The absorbance was measured at 570 nm using a microplate reader (Molecular Devices, Menlo Park, CA, USA). The content of nitrite was calculated based on a standard curve constructed with $NaNO_2$ at the concentrations of 400, 200, 100, 50, 25, 12.5, 6.25 and 3.12 µM (Gómez et al., 2013). All of the reagents used in the cell culture experiments were prepared in the growth medium mentioned above and acquired from Sigma (Dorset, UK).

We conducted a live/dead cell viability assay (Invitrogen, UK) to evaluate the effects of RV, ACh and laminar flow before and after the incubation. The samples were washed for five minutes in PBS and incubated in a solution of 2 µM calcein-AM (live) and 4 µM ethidium homodimer-1 (dead) solution in PBS for 30 minutes. We determined cell viability with the Presto Blue assay (Invitrogen, UK), which quantifies metabolically active cells, according to the manufacturer's instructions. To measure the fluorescence intensity of the assay, a microplate reader (Molecular Devices, Menlo Park, CA, USA) was used. After rinsing, the samples were imaged with an inverted fluorescence microscope (Leica DMI6000B; Leica Microsystems, Germany) using a

conventional fluorescein long pass filter. The quantification of the cells was done with Image J software (US National Institutes of Health).

Statistical Analysis

The dilator responses elicited by ACh and intraluminal flow in the presence of RV or PSS are expressed as percentage relaxation corresponding to the increase from the pre-constricted value. Concentration response curves from the segments of the femoral artery in the pressure myograph and the cultured cells were assessed, independently, using a two-way repeated measures analysis of variance (ANOVA) with as within factors flow (three levels: 5, 8 and 10 $\mu \cdot min^{-1}$) and time, and as between factors RV, age, and/or treatment. If main effects or interactions were found subsequent unpaired t-tests were performed to locate the differences. Values of $p<0.05$ were considered significant. Data are represented as means ± standard error of mean (SEM) unless stated otherwise. Power calculations were not conducted *a priori* for this study.

Results

RV purity and stability

The average calculated purity of RV from the three pills extracted in duplicate was (mean ± SD) 97 ± 13 % (Figure 19). The variability between duplicates is more likely due to handling errors than differences in purity between individual pills. We determined that the concentration of RV at the beginning of the experiments with pressure myography was (mean ± SD) 45 ± 1 μM. The concentration dropped to (mean ± SD) 35 ± 0.1 μM following the 1-hour circulation in the myograph chamber (Figure 20). Hence, there was a loss of 23% between pre- and post-incubation samples. Whilst the samples registered additional early eluting material (most likely predominantly ascorbate), no obvious stilbenoid metabolite peaks were identified.

Constrictor responses to KPSS and Phe

The arteries used in these studies had an initial mean diameter of 138 ± 46 μm (YC; n=11) and 177 ± 31 μm (YRV; n=6), and 150 ± 34 μm (OC; n=6) and 172 ± 38 μm (ORV; n=6). We observed no significant differences in lumen diameter between groups. Incubation with RV had no significant effects on resting lumen diameter. The

segments treated with RV developed no myogenic tone or increase in diameter after perfusion for one hour with intraluminal pressure of 60 mmHg (YRV: 171 ± 19 μm, -2 ± 9 % change; ORV 147 ± 31 μm, -17 ± 18 % change). The degree of constriction to KPSS (YRV: 77 ± 28 μm, -52 ± 17 % change *vs*. ORV: 46 ± 21 μm, -71.1 ± 12 % change) and Phe 10^{-5} M (YRV: 71 ± 25 μm, -58 ± 13 % change *vs*. ORV: 57 ± 20 μm, -67 ± 7 % change) was similar between groups. Figure 21 shows the constrictor responses to KPSS and Phe 10^{-5} M of the isolated pressurized mouse femoral artery following incubation with RV.

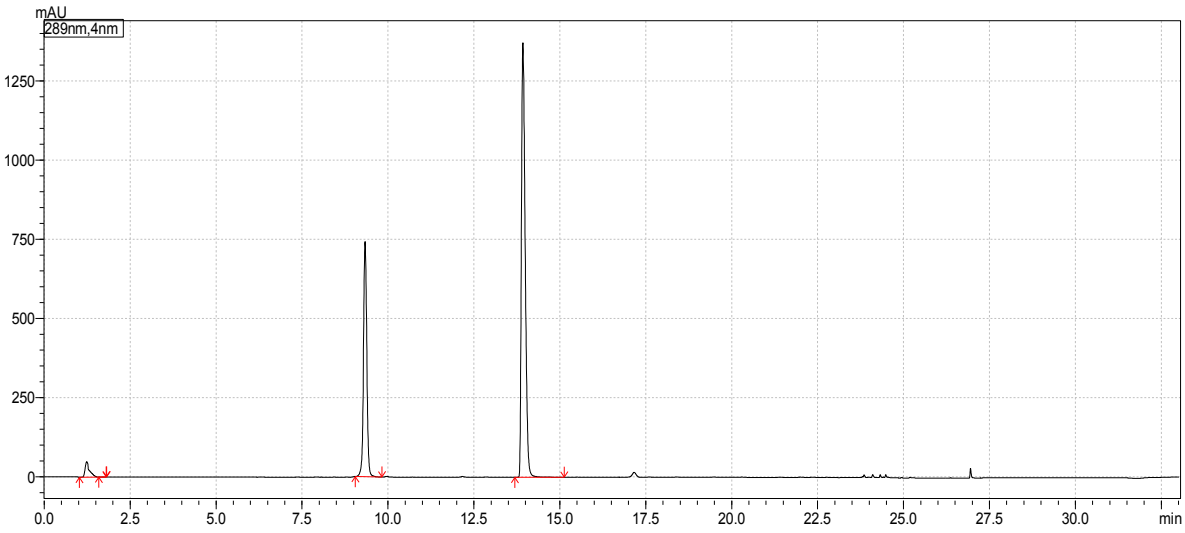

FIGURE 19. PURITY OF RESVERATROL.

Chromatogram of RV primary extract test material monitored at 289 nm showing retention times for taxifolin (reference standard) at 9.3 minutes and for RV at 13.9 minutes. The average calculated purity of RV was (mean ± SD) 97 ± 13. No other peaks other than taxifolin and *trans*-RV were identified in the samples.

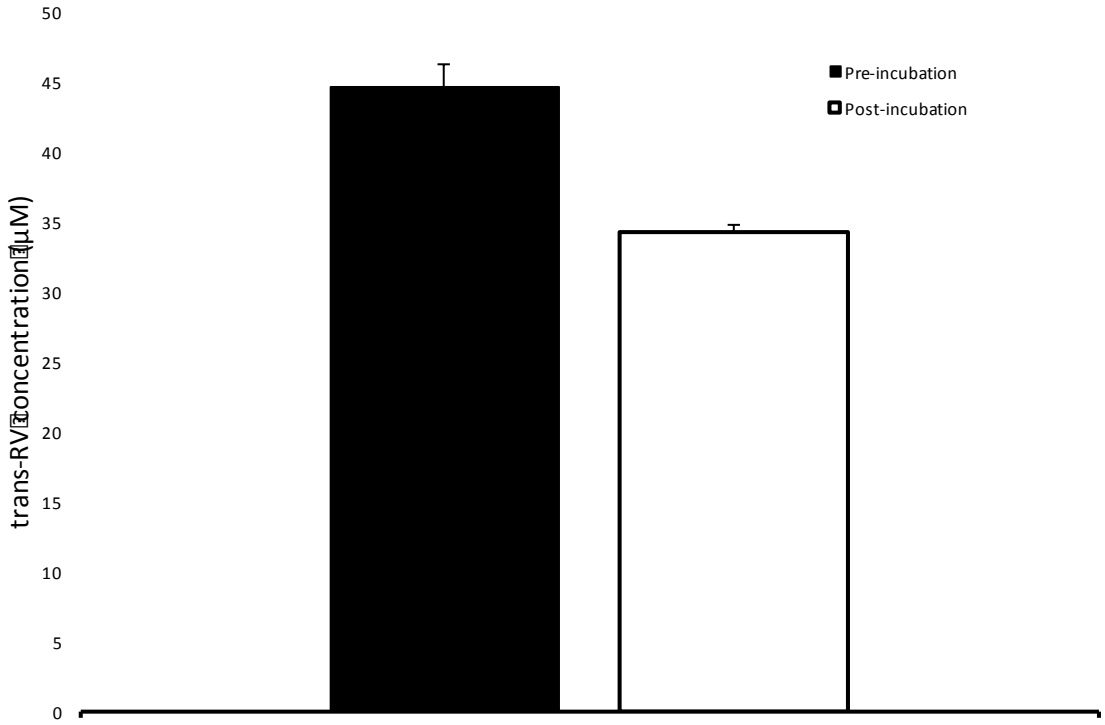

FIGURE 20. STABILITY OF RESVERATROL.

The concentration of RV at the beginning of the experiments with pressure myography was (means ± SD) 45 ± 1 μM. The concentration dropped to 35 ± 0.1 μM following the 1-hour circulation in the myograph chamber. Hence, there was a loss of 23% between pre- and post-incubation samples.

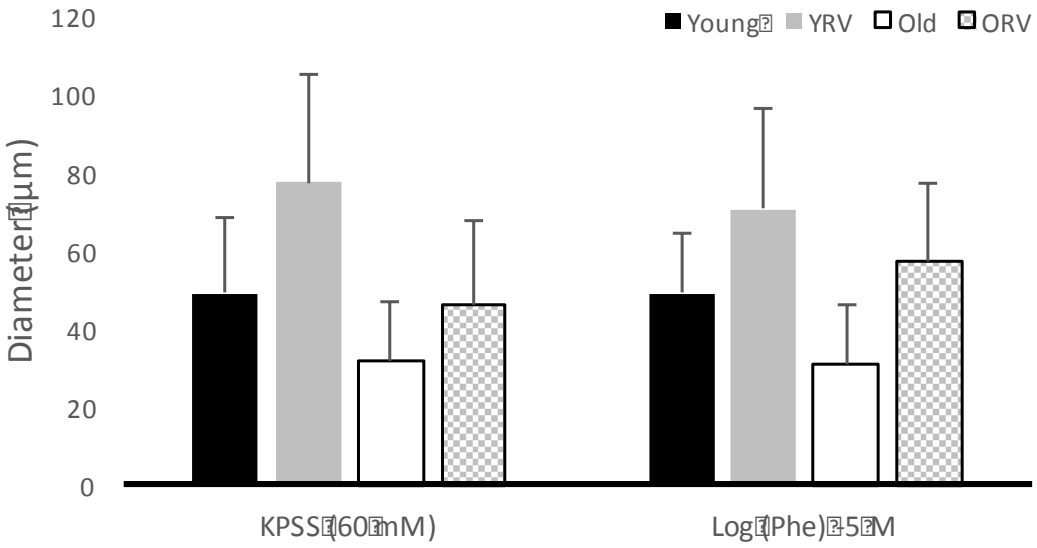

FIGURE 21. CONSTRICTOR RESPONSES TO KPSS AND PHE.

KPSS (60 mM)- and Phe (10^{-5} M)-induced constriction of the isolated pressurized femoral artery from 4-(young) and 26-month old mice (old). The segments of the femoral artery were incubated for one hour with either PSS (young; *n* = 11 and old; *n* = 6) or RV (45 μM; *n* = 6 per group). We observed no effect of RV on KPSS- or Phe-induced constriction in relation to controls. Young RV (YRV); Old RV (ORV). Data are means ± SEM.

Effects of RV on ACh-induced dilation

We observed significant effects of RV on ACh-induced dilation in both groups when compared with their respective age-matched controls ($p<0.05$). RV improved ACh-induced dilation at doses of 10^{-5} – 10^{-3} M. These effects were similar between the young and old. Figure 22 shows the dilator responses to ACh of young and old femoral segments incubated with RV.

Characterization of the ACh-induced dilation

Figure 23 shows the dilator responses of the isolated pressurized young mouse femoral artery to ACh in the presence of inhibitors of endothelial factors and RV. Co-incubation of LNNA or apamin + Tram 34 with RV had no effect on lumen diameter when compared to RV alone or controls. However, co-incubation of indomethacin with RV led to significant increases in diameter when compared to controls ($p<0.05$), but this was not significantly different from that achieved with RV alone.

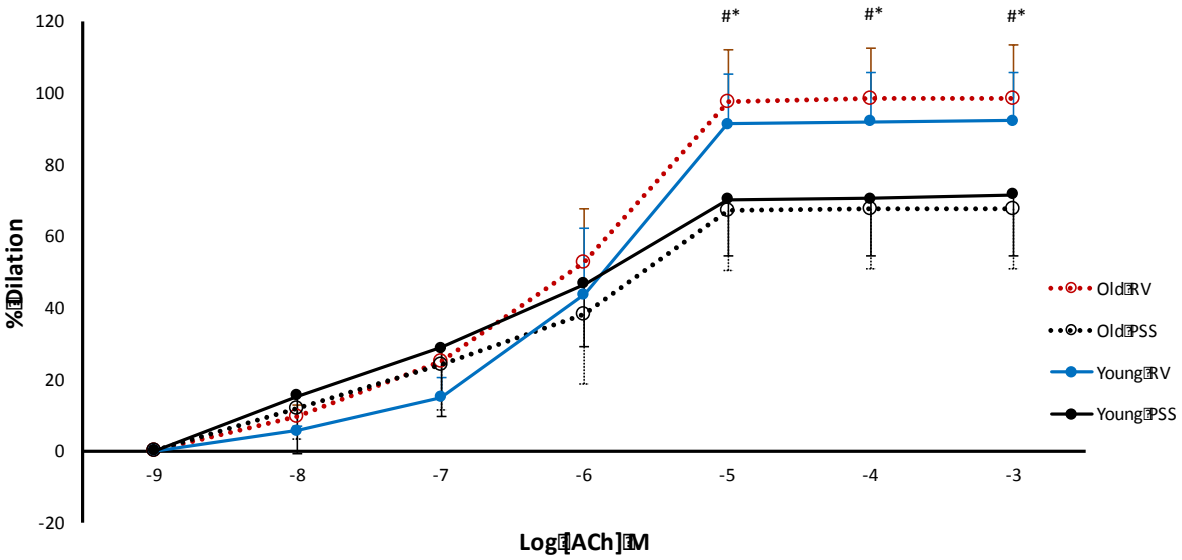

FIGURE 22. EFFECTS OF RV ON ACH-INDUCED DILATION.

RV (45 μM; *n* = 6 per group) significantly improved dilation of the isolated pressurized femoral artery from 4-(young) and 26-month old mice in response to ACh 10^{-5} – 10^{-3} M when compared to age-matched controls (young: *n* = 11; old: *n* = 6) ($p<0.05$). These effects were similar between the young and old. # Young; Different from Young RV at $p<0.05$. * Old; Different from Old RV at $p<0.05$. Data are means ± SEM.

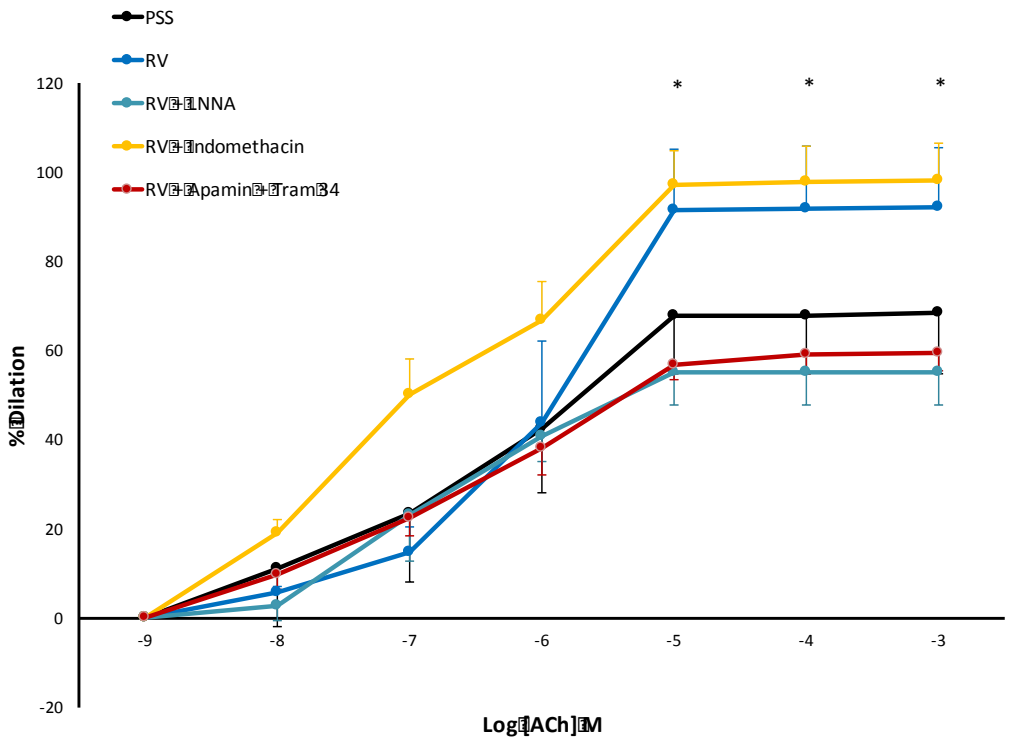

FIGURE 23. CHARACTERIZATION OF THE DILATOR RESPONSE TO ACH IN THE PRESENCE OF RV. Co-incubation of RV (45 μM) with LNNA (100 uM; *n* = 5) or apamin (100 nM) + Tram 34 (1 uM) (*n* = 5) had no effect on ACh (10^{-9}-10^{-3} M)-induced dilation of the isolated pressurized femoral artery from 4-month old mice when compared to age-matched controls (PSS; *n* = 11). However, when compared to controls co-incubation of RV with indomethacin (10 uM; *n* = 5) led to significant increases in diameter ($p<0.05$). Data are means ± SEM. * Treatments are different from RV and the combination of RV and indomethacin at $p<0.05$.

Effects of RV on FMD

Incubation with RV significantly reduced FMD to negligible levels in the young and old ($p<0.05$). Figure 24 shows the dilator responses to intraluminal flow in femoral segments incubated with RV.

Characterization of maximal FMD

Co-incubation of RV with LNNA, apamin + Tram 34 or indomethacin significantly reduced maximal FMD ($p<0.05$). The levels of maximal FMD caused by the co-incubation of RV with the inhibitors of endothelial factors were of similar magnitude than those observed with RV alone (Figure 25).

Effects of RV on the sustained response to intraluminal flow

We found a significant main effect of RV on sustained dilation (the initial maximal dilation *vs*. the sustained dilation prior cessation of flow; $p<0.05$). The two-way ANOVA revealed a significant interaction between RV and condition ($p<0.05$), which indicates that the effect of inhibitors differs between controls and segments

incubated with RV and 2) an interaction between initial maximal and sustained diameters with condition ($p<0.05$), which suggests that the effect of the inhibitors differs at different time points. Together, these results indicate that in response to intraluminal flow, RV on its own and in combination with inhibitors of endothelial factors, significantly and negatively affects the capacity of the vessel to auto-regulate diameter. Figures 26-27 show the effects of RV on sustained diameter of the isolated mouse femoral artery in response to intraluminal flow.

Effects of RV on NO production

We replicated the incubation with RV and time responses to dilator stimuli of the pressure myograph in cultured EC. We observed that the production of NO in response to ACh is the highest during the first two minutes with a steady decrease over time ($p<0.05$). The two-way ANOVA revealed a significant effect of time on the production of NO. Incubation with RV significantly reduced the production of NO at two minutes. However, it significantly increased it following incubation with ACh for one hour when compared to controls ($p<0.05$; Figure 28). We found very similar results in response to flow. The production of NO decreases over time ($p<0.05$) and is significantly less after incubation with RV ($p<0.05$; Figure 29). Incubation with RV had no negative effects on cell viability (Figure 30). Cell viability amongst all groups was (mean ± SD) 96 ± 2%.

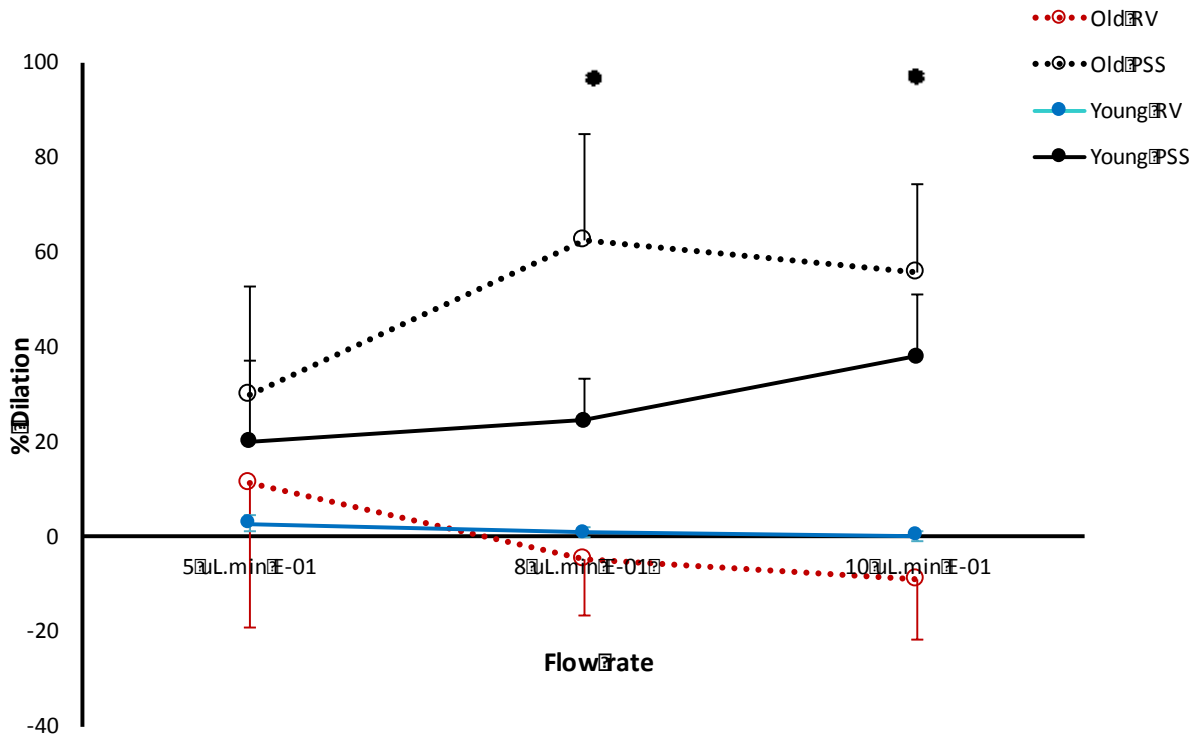

FIGURE 24. EFFECTS OF RV OF THE DILATOR RESPONSE TO INTRALUMINAL FLOW.

Incubation with RV (45 μM) significantly reduced the dilator response of the isolated pressurized femoral artery from 4-(young; *n* = 6) and 26-month old mice (old; *n* = 6) to intraluminal flow (8 and 10 μL·min^{-1}) when compared to age-matched controls (PSS; young; *n* = 11 and old; *n* = 6) ($p<0.05$). # Young; Different from Young RV at $p<0.05$. * Old; Different from Old RV at $p<0.05$. Data are means ± SEM.

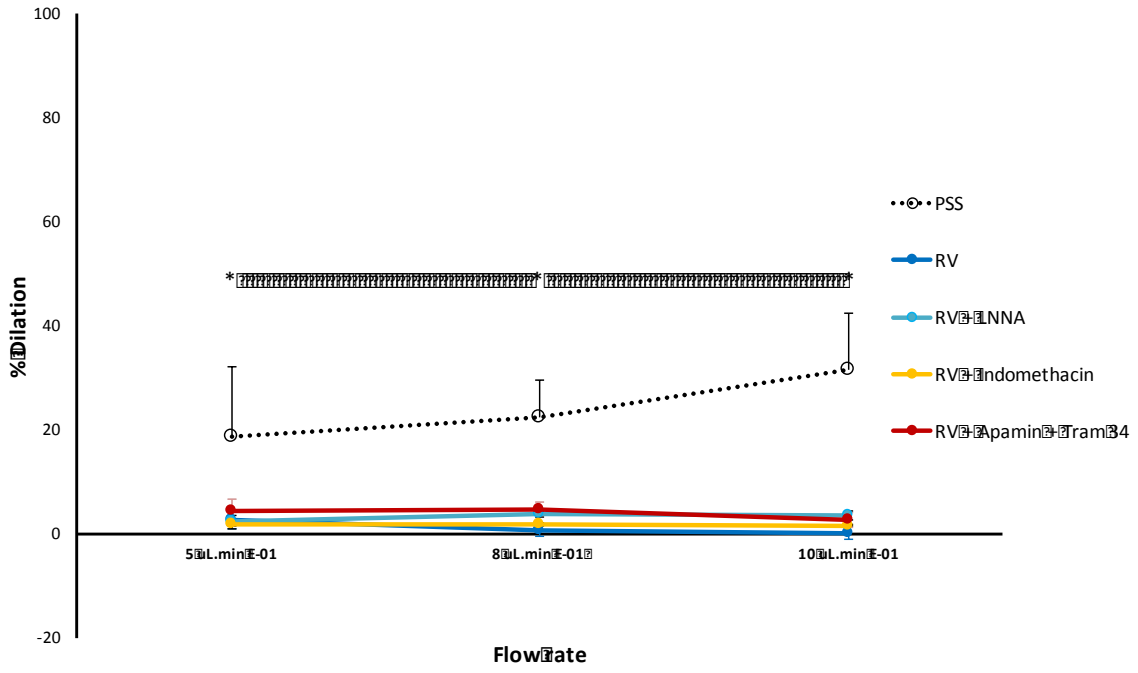

FIGURE 25. CHARACTERIZATION OF THE DILATOR RESPONSE TO INTRALUMINAL FLOW IN THE PRESENCE OF RV.

Co-incubation of RV (45 μM) with LNNA (100 uM; *n* = 5), apamin (100 nM) + Tram 34 (1 uM) (*n* = 5) or indomethacin (10 uM; *n* = 5) significantly reduced flow (5-10 μL·min^{-1})-mediated dilation of the isolated pressurized femoral artery from 4-month old mice when compared to age-matched controls (PSS; *n* = 11) ($p<0.05$). Data are means ± SEM.

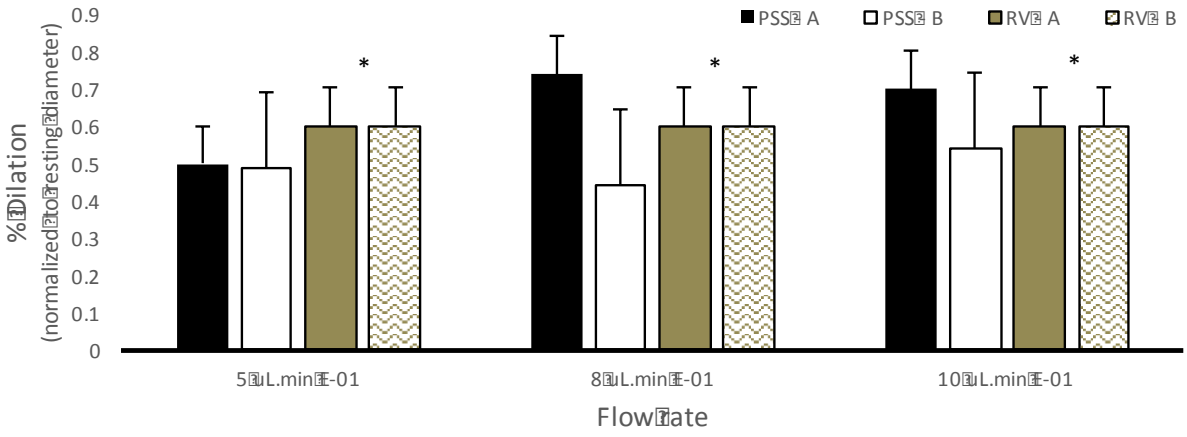

FIGURE 26. EFFECTS OF RV ON THE SUSTAINED FMD OF ISOLATED PRESSURIZED FEMORAL ARTERIES FROM YOUNG MICE.

Incubation of isolated pressurized femoral arteries from 4-month old mice with RV (45 uM; *n* = 6) significantly reduced the capacity of the vessel to auto-regulate tone in the presence of intraluminal flow (5-10 $\mu L \cdot min^{-1}$). The ratio between the maximal diameter upon introduction of flow to the sustained diameter prior cessation of flow was significantly lower in the presence of RV ($p<0.05$). A- maximal diameter upon introduction of flow. B – sustained diameter prior cessation of flow. * Different from PSS at $p<0.05$. Data are means ± SEM.

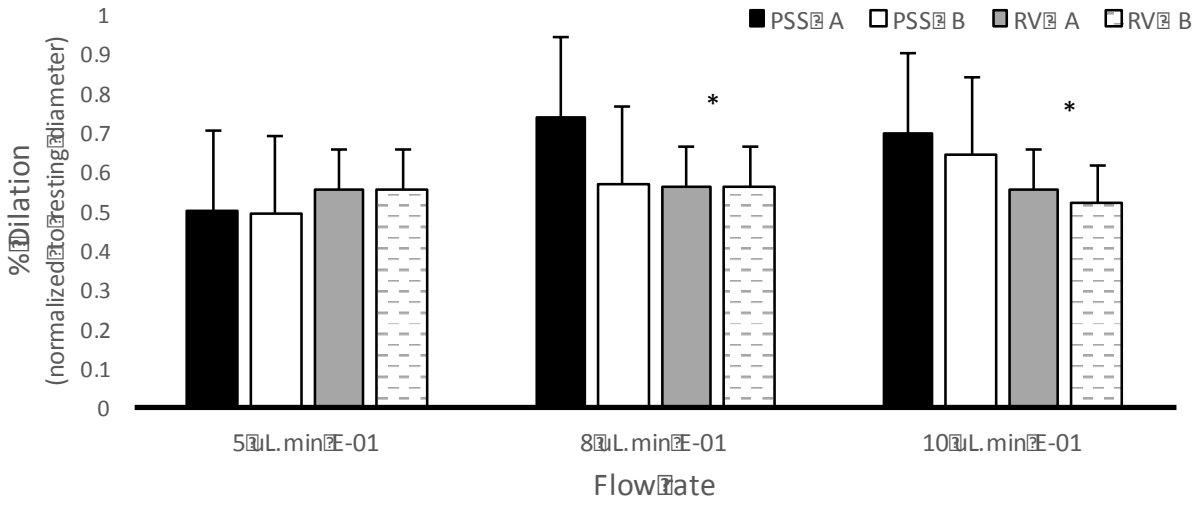

FIGURE 27. EFFECTS OF RV ON THE SUSTAINED FMD OF ISOLATED PRESSURIZED FEMORAL ARTERIES FROM OLD MICE.

Incubation of isolated pressurized femoral arteries from 26-month old mice with RV (45 uM; *n* = 6) significantly reduced the capacity of the vessel to auto-regulate tone in the presence of intraluminal flow (5-10 $\mu L \cdot min^{-1}$). The ratio between the maximal diameter upon introduction of flow to the sustained diameter prior cessation of flow was significantly lower in the presence of RV ($p<0.05$). A- maximal diameter upon introduction of flow. B – sustained diameter prior cessation of flow. * Different from PSS at $p<0.05$. Data are means ± SEM.

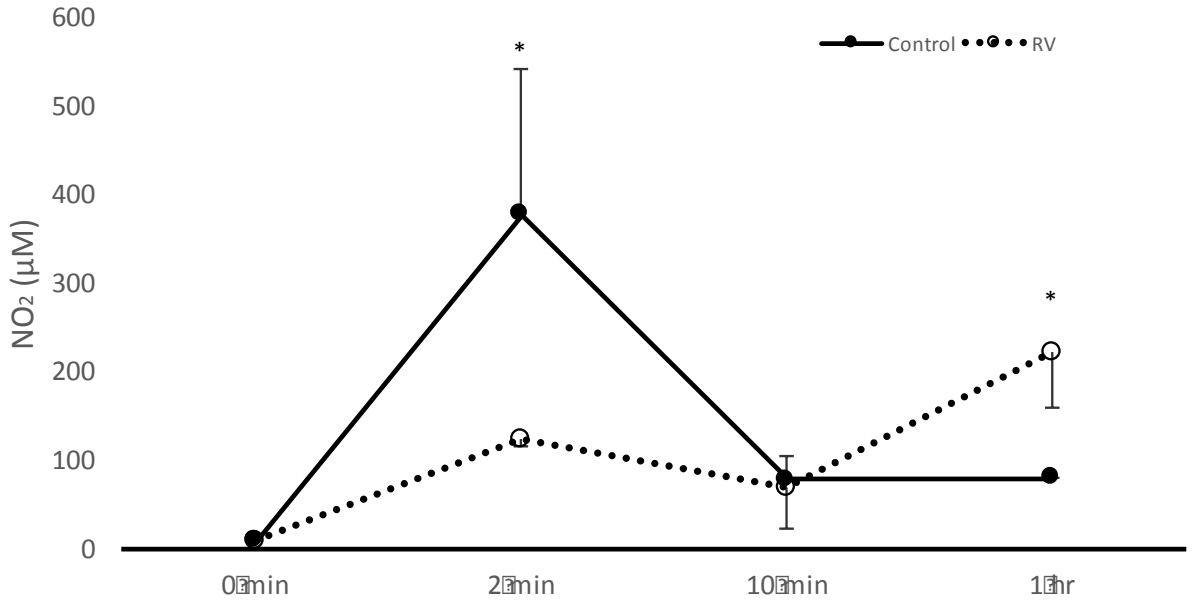

FIGURE 28. EFFECTS OF RV ON ACH-INDUCED NO_2 PRODUCTION BY CULTURED HCAEC.

Pre-incubation with RV (45 μM) for one hour reduced the production of NO_2 by cultured HCAEC in response to ACh (10^{-5} M) for two minutes when compared to controls ($p<0.05$). However, when the cells were exposed to ACh for one hour, RV significantly increased the production of NO_2 ($p<0.05$). * different from controls at $p<0.05$. Data are means ± SEM.

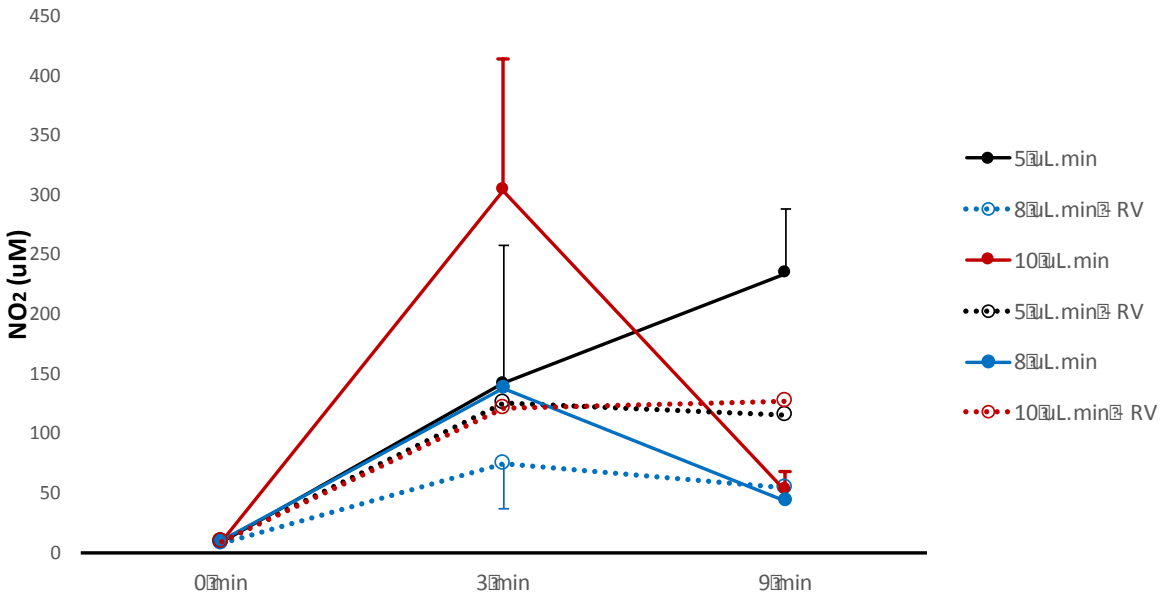

FIGURE 29. EFFECTS OF RV ON FLOW-INDUCED NO_2 PRODUCTION BY CULTURED HCAEC.

Pre-incubation with RV (45 µM) for one hour reduced the production of NO_2 by cultured HCAEC in response to laminar flow (5-10 $\mu L \cdot min^{-1}$), particularly during the first three minutes of exposure under 8 and 10 $\mu L \cdot min^{-1}$ ($p<0.05$). Data are means ± SEM.

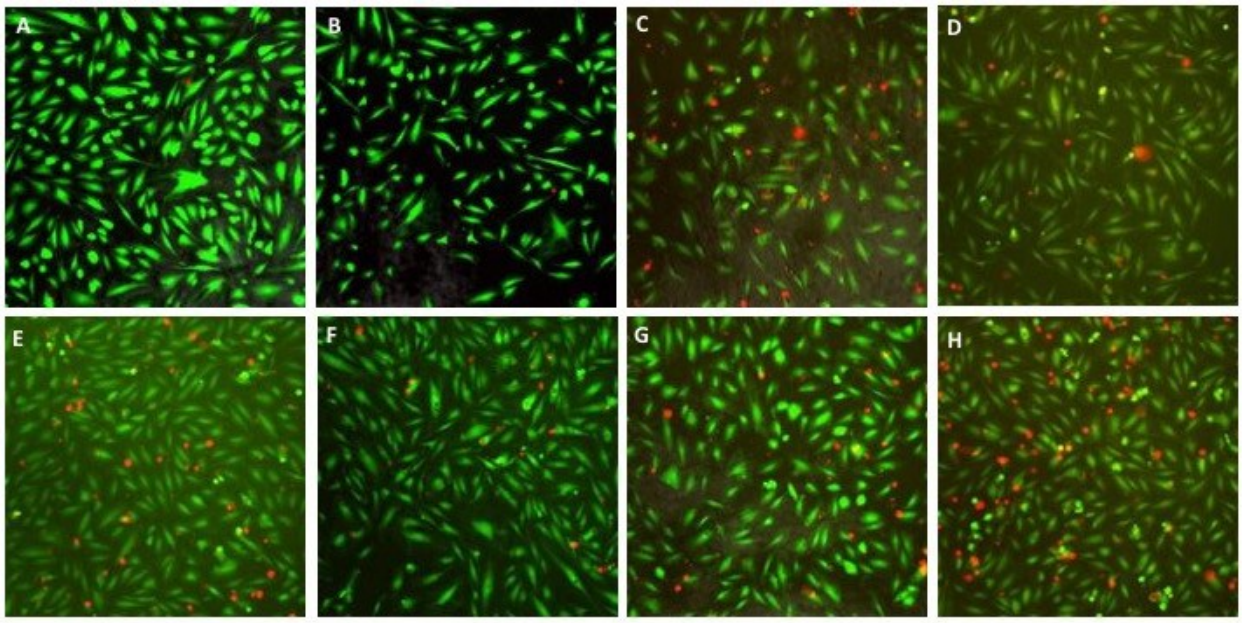

FIGURE 30. LIVE-DEAD ASSAY OF CULTURED HCAEC.

Representative images of the HCAEC Live-Dead assay. Cultured cells were incubated with growth medium (A) or RV (45 uM) for one hour (B). Following incubation, the cells were exposed to ACh for 2 (C), 10 (D), and 60 minutes (E) with ACh (10^{-5} M). A different group of cells were exposed to laminar flow (5-10 $\mu L \cdot min^{-1}$) for three (F; 5 $\mu L \cdot min^{-1}$), six (G; 8 $\mu L \cdot min^{-1}$) and nine minutes (H; 10 $\mu L \cdot min^{-1}$). Dead cells were stained with EthD-1 (red); live cells were stained with Calcein-AM (green). No treatment (ACh, laminar flow or RV) had negative effects on cell viability in relation to controls. Cell viability amongst all groups was (mean ± SD) 96 ± 2%.

Discussion

The main findings of this study are that 1) within the setting of pressure myography *trans*-RV is stable (no conversion to *cis*-RV any other metabolite is apparent) and is the most likely candidate to exert the observed vascular effects, 2) *ex-vivo* RV causes differential effects on ACh-induced and FMD, 3) *in-vitro* RV reduces the production of NO in response to ACh or laminar flow during the first 10 minutes. However, it enhances NO production upon incubation with ACh for one hour. These findings uncover important methodological and physiological issues regarding the potential of RV to improve vessel dilation; 1) with few exceptions, the vast majority of studies investigating the effects of RV on vascular parameters have not determined the molecule to which the target cell/tissue is exposed. This information is necessary to properly understand and methodically compare published findings; 2) Differential effects on ACh-induced and FMD suggest RV might affect flow-mediated endothelial mechanotransduction, which makes the comparison between data gathered from pressurized ACh dose-responses and intraluminal flow models problematic; 3) The adverse effects of RV on FMD might be partially explained by a compromised production of NO.

RV stability

Recent studies *in-vivo* have challenged the beneficial effects of RV on the vasculature (Yoshino et al., 2012; Gliemann et al., 2013; Poulsen et al., 2013; Gliemann et al., 2014). Despite low bioavailability in humans after digestion (Walle et al., 2004; Walle, 2011), the discrepancies between lines of experimentation seem not to be exclusively associated with the dose or the duration of treatment. Rather, some have attributed the lack of effects of RV *in-vivo* to the metabolic characteristics of the population investigated suggesting that improvements might only be observed in subjects whose health is significantly compromised (Olesen et al., 2014). Surprisingly, little attention has been paid to RV metabolism when discussing such findings. It has been reported that in mammals RV undergoes extensive phase I and phase II metabolism (Monika et al., 2016), including conjugation with sulphate groups and glucuronic acids. Sulfation and glucuronidation reduce the cell permeability to certain molecules, such as RV (Monika et al., 2016). Hence, it is reasonable to think that RV metabolites do not exert the same degree of physiological effects *in-vivo* as non-metabolized RV does *in-vitro* or *ex-vivo* (Gescher and Steward, 2003). In the present study, we have shown that in settings of pressure myography, the effects on ACh-induced and FMD of the isolated pressurized mouse femoral artery are most likely mediated by non-metabolised *trans*-RV. Following incubation for 60 minutes, 23% of RV is lost from the bath solution. Such degradation is most likely a function of oxidation (Vian et al., 2005) due to circulating the solution through the system with the pool open to air

since the HPLC analysis revealed no other peaks for *cis*-isomers or RV metabolites. Even though we cannot rule out isomerization per se since we did not use any *cis* standard for reference, the only possibility isomerization accounted for RV degradation would be if the chromatography employed did not separate the isomers or if the response factor for the *cis*-isomer was significantly lower at the same wavelength.

ACh-induced dilation

The present study has defined differential effects of RV on ACh-induced and FMD of the isolated pressurized mouse femoral artery. Regardless of age, RV markedly improved ACh-induced dilation. Unexpectedly, however, it drastically compromised FMD. In cardiovascular physiology, most studies *ex-vivo* have used agonists (i.e., Phe and ACh) to study vessel responses. Organ baths and wire myographs, for instance, represent a convenient model to investigate dose responses in vessels independently of systemic influences. However, it is not possible to pressurize or introduce flow into the vessels. Although the study of vessel responses with the use of chemical instead of mechanical stimulus provides very important information regarding the mechanisms underlying vessel dilation, FMD simulates more closely the natural functioning of the vessel. Insight into important mechanisms regarding mechanotransduction, for instance, is lost with the sole use of agonists. In regards to ACh-induced dilation, when we compare our findings to those investigating the

vasorelaxant effects of RV on vessels in the absence of intraluminal flow, they are broadly consistent. RV (5-50 μmol·L and 15 mg·kg for two weeks) has been shown to induce concentration-dependent vasodilation in noradrenaline- and potassium chloride- pre-constricted mesenteric and uterine arteries from female guinea-pigs (Naderali et al., 2000) and mesenteric arteries of male F344 x Brown Norway rats (Gocmez et al., 2016), respectively. Further, RV (5-35 μmol·L) caused similar levels of dilation in the mesenteric arteries of lean and obese Wistar rats (Naderali et al., 2001). In that study, ACh-induced dilation was blunted in the arteries from obese rats, which led the authors to conclude that RV might also promote dilation by means of endothelium-independent mechanisms (Naderali et al., 2001). In support of this, RV (5 mg·kg·day for six weeks and 0.48 mg·L for four weeks) restored the ACh-induced dilation of the mesenteric arteries and the aorta, respectively, from young spontaneously hypertensive rats (Rush et al., 2007; Bhatt et al., 2011). The mechanisms involved appeared to be attenuation of oxidative stress, prevention of eNOS uncoupling and increased expression of critical proteins regulating the synthesis of NO, such as eNOS and sGC (Bhatt et al., 2011). Similar to our study, some reports have found no influence of vasodilator prostanoids on RV-induced dilation as no variation in lumen diameter was observed in the presence of indomethacin (Naderali et al., 2000; Rakici et al., 2005).

Flow-mediated dilation

As mentioned above, FMD is considered a clinical measure of endothelium-dependent dilation (Heiss et al., 2015) and as such, it offers valuable physiological insight. Since most studies *ex-vivo* have used agonists or non-pressurized vessels, it has been generally assumed that RV enhances vessel dilation in response to intraluminal flow in similar fashion than in response to ACh. However, the fact that in our study RV improved ACh-induced dilation yet it compromised FMD, suggests the two processes are differentially regulated. The mechanisms mediating such effects are likely to involve the inhibition of endothelial factors since co-incubation with LNNA, apamin + Tram 34 or indomethacin resulted in significantly lower levels of dilation when compared to controls. In addition to this, RV significantly compromised the capacity of the isolated pressurized femoral artery to regulate diameter in the constant presence of flow. We are not aware of any other study *ex-vivo* that investigated the effects of RV on FMD in the isolated pressurized mouse femoral artery. Hence, it is difficult to compare our findings. However, some evidence exists *in-vivo*. Although a recent meta-analysis indicated that RV does not reduce BP regardless of dose or duration of treatment, or the BMI of the subjects (Liu et al., 2015), it seems to have positive effects on FMD. For instance, RV (3 mg·kg·day for six weeks) is reported to improve endothelial function of the femoral artery in rabbits fed a high-cholesterol diet (Zou et al., 2003). Further, in middle-aged and older obese humans with elevated BP, but not in normotensive subjects, acute (30-270 mg) and chronic (75 mg·day for

six weeks) supplementation with RV improved FMD of the brachial artery (Wong et al., 2011; Wong, Berry, et al., 2013). Because in these studies RV was given orally, and thus it was subjected to digestion and absorption, it is unfortunate that the authors did not identified the exact constituent responsible for such effects.

The differential effects of RV on ACh-induced and FMD constitute a powerful impetus for the further investigation of the cardiovascular properties of RV. Several aspects of these findings are unique. Consequently, we have decided to adopt a more conservative approach regarding their interpretation. Although speculative, divergent effects in response to ACh and flow suggest RV might influence flow-mediated endothelial mechanotransduction. Further experiments are necessary to test this hypothesis. More importantly perhaps, our findings also raise very important questions with respect to the information gained from *ex-vivo* models. Ultimately, the translational potential of RV is only valid if proven beneficial under physiological conditions. The disagreement between lines of experimentation might rely too on the transduction of chemical and mechanical signals from the endothelium to the vascular smooth muscle. RV might induce, *in-vitro*, the expression of critical transcription factors thought to confer a vasoprotective phenotype (Gracia-Sancho et al., 2010). However, such molecular responses must be properly translated into physiological adaptations in order to be of value. If in fact, RV compromises the transduction of mechanical signals in pre-constricted pressurized vessels subjected to intraluminal flow, our findings could have a profound impact on the interpretation

of data from *in-vitro* and some *ex-vivo* experimental models. The RV-induced inhibition of mechanotransduction, if true, would render rather irrelevant the data accumulated from these models, particularly those using isolated, but non-pressurized vessels or pressurized vessels in which FMD is not investigated (Gescher and Steward, 2003). Thus, considering the stark discrepancy between the effects of RV on ACh-induced and FMD, it is imperative to determine the mechanism by which RV influences flow-mediated endothelial mechanotransduction.

On the other hand, RV is a known scavenger of $O^{2}\bullet^{-}$ and •OH (Leonard et al., 2003; Cao and Li, 2004). In light of the dependence of ROS for vasodilation (Zhang et al., 2012; Chidgey et al., 2016), it could be argued that the dose of RV used in our study inhibited the secretion of important EDHFs, such as H_2O_2 thus compromising FMD. However, the effects of laminar flow on the production of NO by cultured EC following incubation with RV, as discussed below, suggest that RV might inhibit FMD not only by EDHF-dependent mechanisms.

Production of NO

We determined the effects of RV on NO production by HCAEC in response to ACh (10^{-5} M) and laminar flow (5-10 $\mu L \cdot min^{-1}$). The exposure to ACh and laminar flow were of the same duration as in the pressure myograph. Of note, during the first minutes of

exposure to ACh and laminar flow, RV reduced the production of NO. However, when the cells were exposed to ACh for one hour, RV significantly increased NO production. This is in contrast with a previous study that reported RV-induced concentration-dependent increases in the production of NO by EA.hy 926 cells following incubation with RV for two minutes (Wallerath et al., 2002). Other studies that tested the effects of non-metabolized *trans*-RV in the μM (Wallerath et al., 2002) and nM (Diebolt et al., 2001; Klinge et al., 2008; Nicholson et al., 2008) range on the production of NO have found significant increases in its concentration only upon incubation periods of >24 hours. Interestingly, a recent report provided evidence suggesting that *in-vitro* incubation of EA.hy926 cells with *trans*-RV, but not its metabolites, for 24 hours results in elevated eNOS activity and gene expression, NO release from the endothelium and the reduction of intracellular ROS levels (Ladurner et al., 2014). Since the effects of RV on gene transcription and eNOS mRNA stabilization are rather slow (Ladurner et al., 2014), one could argue that *in-vitro* relatively long periods of incubation are necessary to increase NO production by EC.

Our findings on cultured cells provide additional insight into the effects of RV on ACh-induced and FMD of the isolated pressurized mouse femoral artery. Considering the lower levels of NO production by HCAEC during the first 10 minutes of exposure to ACh and laminar flow following RV incubation, one could argue that the effects of RV on ACh-induced and FMD are NO-independent and dependent, respectively. This is better illustrated by the fact that addition of LNNA did not significantly reduced

dilation in response to ACh whereas it drastically compromised it in response to intraluminal flow.

Conclusions

Taking everything into consideration, our findings indicate that in the isolated pressurized mouse femoral artery non-metabolized *trans*-RV causes differential effects on ACh-induced and FMD dilation. Whereas it significantly enhances ACh-induced dilation, it abolishes FMD. RV reduced the production of NO by HCAEC during the first 10 minutes of exposure to either ACh or laminar flow. However, following incubation with RV and in response to exposure to ACh for one hour, the concentration of NO increased significantly. This, together with the fact that addition of LNNA did not have any effects on ACh-induced dilation, but significantly compromised FMD suggest that the effects of RV on ACh-induced and FMD are NO-independent and dependent, respectively. Investigating vasodilation by means of agonists (i.e. ACh) might not provide a comprehensive understanding of the effects of RV on endothelium-dependent dilation. This is particularly important since FMD is considered to be a clinical measure of the latter. One possibility that might reconcile the disagreement between the two models involves the disruption of flow-mediated endothelial mechanotransduction.

Chapter 4

Effects of Acute Supplementation with Resveratrol on Flow-mediated Dilation and Oxygen Consumption Kinetics in Older Coronary Artery Disease Patients

Abstract

Coronary artery disease (CAD) is the main cause of death worldwide. The major underlying cause of CAD is atherosclerosis of the coronary arteries. Resveratrol (RV) is a polyphenol naturally found in the skin of grapes, a variety of berries, and peanuts known to improve cardiovascular function *in-vitro* and *ex-vivo.* We investigated the effects of acute supplementation with high doses of RV on flow-mediated dilation (FMD) and oxygen consumption (VO_2) kinetics in older, CAD patients. In a placebo-controlled, single blind, crossover trial 10 participants (aged 67.7 ± 7.0 years) received either RV or placebo (1 g · day) during three consecutive days plus additional 330 mg in the morning of the fourth day with a seven-day wash-out period in-between. On the fourth day, FMD of the brachial artery and muscle oxidative capacity by means of VO_2 kinetics were determined. RV had no effect on the resting diameter of the brachial artery or on endothelium-dependent dilation. Likewise, we found no significant effect of RV on VO_2 kinetics, time constant, and mean response time. Our findings question previous promising data obtained from murine and *in-vitro* studies and suggest no significant benefits from acute and high doses of RV on cardiovascular function of older CAD patients.

Keywords: Antioxidant; Ageing; Endothelial Dysfunction; Oxygen Uptake.

Introduction

Coronary artery disease (CAD) is the main cause of death worldwide. The major underlying cause of CAD is atherosclerosis of the coronary arteries (Bruning and Sturek, 2015). Patients with CAD commonly present with compromised endothelial function, which is characterized by a compromised capacity of the vessel to dilate in response to endothelium-secreted vasodilators, mainly nitric oxide (NO) (Currie et al., 2014). Flow-mediated dilation (FMD) of the brachial artery is a well-established method to estimate endothelial function (Currie et al., 2014), which has been demonstrated to be lower in CAD patients than in healthy age-matched controls (Neunteufl et al., 1997). In addition to compromised endothelial function, the presence of atherosclerotic plaques in the coronary arteries may reduce blood flow, and hence, oxygen delivery to the myocardium even when heart rate and systolic blood pressure (BP) are elevated (Bruning and Sturek, 2015). These conditions are exacerbated in old age as consequence of the natural decline of the cardiovascular system with time.

Atherosclerotic plaques and compromised endothelial function can also have a significant impact on skeletal muscle function. An increase in power output by the skeletal muscle requires a proportional increase in the production of ATP (Buckley and Bossen, 2013). Even though during the initial seconds in the transition from rest to work the muscle can rely on ATP from the breakdown of phosphocreatine and

glucose, prolonged work can only be performed when the aerobic generation of ATP can meet such demands. This requires an adequate supply of oxygen to the mitochondria in the muscle cell, and hence, an adequate blood flow through the muscles (Korzeniewski and Zoladz, 2006). Because dilation of the vessels is impaired, oxygen and nutrient supply to both the skeletal muscle and the myocardium is not adequate in CAD patients (Bruning and Sturek, 2015). Consequently, they present lower rates of oxygen consumption (VO_2) and aerobic capacity during exercise (Bruning and Sturek, 2015).

Resveratrol (RV) is a polyphenol naturally found in the skin of grapes, a variety of berries, and peanuts. RV went unnoticed until the 1990s when it was related to the "French Paradox", a term used to describe a low incidence of coronary heart disease in France despite the high dietary intake of cholesterol and saturated fat. This phenomenon was explained by the regular intake of red wine and in particular, RV and other polyphenols (Liu et al., 2007).

A significant body of research has demonstrated that in rodents and in models *in vitro,* RV has anti-oxidant, anti-inflammatory, anti-carcinogenic and cardio-protective properties. Its effects are attributed to the activation of PGC-1α, and SIRT1, both key regulators of oxidative metabolism and mitochondrial biogenesis as well as the down-regulation of the trigger of pro-inflammatory responses, NF-κB (Lagouge et al., 2006; Ungvari et al., 2009). For instance, RV has been shown to reduce oxidative

damage in the aorta of aged rats by down-regulating NADPH oxidase while increasing the expression of SIRT1 (Tang et al., 2012). In endothelial cells, RV has been shown to significantly increase the synthesis of NO and the protein levels of eNOS (Leikert et al., 2002). When combined with regular exercise, RV increased oxygen consumption and mRNA content of oxidative enzymes in the skeletal muscle of senescence-accelerated mice (Murase et al., 2009). However, thus far conflicting results have been reported in humans. RV has been shown to enhance insulin sensitivity, increase the levels of intramyocellular lipids, and reduce intrahepatic lipid content and BP in adult males with type 2 diabetes (Brasnyó et al., 2011). In overweight men with high BP, acute supplementation with RV improved FMD, which was correlated with blood RV levels (Wong et al., 2011). On the other hand, other studies have reported that RV blunts the positive effects of regular exercise in healthy young (Scribbans et al., 2014) and elderly men (Gliemann et al., 2013; Olesen et al., 2014). Although still very limited, these findings cast doubt on the therapeutic effects of RV in humans. Important questions such as age of the population to be treated, and the duration of supplementation and dose of RV still need to be comprehensively addressed.

Considering the effects of RV on endothelial function and VO_2, we reasoned that acute supplementation with a high doses of RV would improve FMD and VO_2 kinetics during low-intensity exercise in CAD elderly patients. Due to the higher levels of inflammation (Kirbis et al., 2010), oxidative stress (Alexander, 1995) and cardiovascular risk of elderly CAD patients, there is reason to believe that

supplementation with RV would be highly beneficial. Further, since the majority of CAD-related morbidities is caused by modifiable behaviour, such as eating and exercise (Bruning and Sturek, 2015), a simple nutraceutical strategy such as supplementation with RV might bear considerable potential in decreasing the prevalence of CAD. In this study, we aimed to determine to what extent FMD and oxygen consumption can be improved with RV in the brachial artery of CAD elderly patients.

Study design and population

After approval by the medical ethical committee of UZ Leuven/KU Leuven, CAD patients who participated in a phase III cardiac rehabilitation program were asked to participate in a placebo-controlled single-blind, crossover, dietary intervention trial comprising three visits to the laboratory within three weeks. The trial was conducted at the University Hospital Leuven (Leuven, Belgium). Patients were included if they were between 45 to 75 years of age, had a history of CAD (post-percutaneous coronary intervention, post-myocardial infarction, or post-coronary artery bypass graft), were on optimal medical treatment and stable with regard to symptoms and pharmacotherapy for at least six weeks. Patients were determined eligible to participate only after approval of their cardiologist. Exclusion criteria for the study included: significant undercurrent illness in the last six weeks, known severe ventricular arrhythmia with functional significance, significant exercise-induced

myocardial ischemia or arrhythmia, or any other disease that would limit exercise performance, the use of RV or antioxidant supplements within the three months prior to the study and consumption of more than three drinks of alcohol per week. Smoking was allowed. A total of 14 patients were screened and selected fit to participate in the study. However, only 10 patients completed all measurements. The patients who did not adhere to the study cited conflicting schedules to attend all physiological measurements as the main reason for it.

Methods

Eligible patients were contacted during the maintenance phase of the cardiac rehabilitation program (phase III) and were informed about the study. Participating patients provided written informed consent, according to the principles of Good Clinical Practice and the Declaration of Helsinki. After giving written informed consent, the patients attended the laboratory on three different occasions. For the first visit, the patients performed a screening maximal cardiopulmonary exercise test on a cycle ergometer (Ergometrics 800 S. Ergometrics, Bitz, Baden-Württemberg, Germany). During the test, an initial workload of 20 W was increased by 20 W every minute until volitional exhaustion. A 12-lead electrocardiogram was recorded continuously as well as breath-by-breath gas exchange analysis (Oxycon Pro TM Jaeger, Carefusion 234, GMbH Hoechberg, Germany). Peak VO_2 was defined as the highest 30-s average of VO_2 at the end of the test.

The patients returned to the laboratory on two more occasions: one after supplementation with RV and one after supplementation with placebo. During each of these two visits, the patients underwent measurements of brachial artery endothelial function followed by assessment of VO_2 kinetics. All tests were performed between 08:00 and 10:00 am in a fasted state, one hour after ingestion of the last capsule (RV or placebo, respectively) and with a seven-day wash-out period in-between. None of the patients experienced any adverse effects. All of the patients were familiar with the measurement of brachial artery endothelial function previous to this study. Population characteristics are described in Table 1.

TABLE 2. CHARACTERISTICS OF THE STUDY POPULATION.

Basic Characteristics	Mean ± SD or Number
Gender (M/F)	9/1
Age (Years)	67.7 ± 7.3
Weight (Kg)	79.1 ± 13.4
BMI (kg/m^2)	26.6 ± 3.7
Maximal VO_2 (mL·min)	2026 ± 655

Supplementation

Both RV (97% pure, *polygonum cuspidatum* extract; 21st Century Alternatives, West Sussex, UK) and placebo (microcrystalline cellulose, 21st Century Alternatives, West Sussex, UK) were given in capsules of 330 mg each. The capsules were identical in appearance and weight and presented in white bottles. Following the screening, the participants received the first bottle containing 10 capsules of either RV or placebo. They were instructed to consume one capsule every eight hours (i.e. three times per day) for three days and to ingest the last capsule one hour before the start of the experimental session in the morning of the fourth day. After the second visit, the participants received the second bottle of pills, which they were instructed to consume in the same regimen three days before the last visit to the laboratory. The participants were blinded to the supplementation. Adherence to the supplementation was monitored by interviewing the participants upon arrival to the laboratory on each visit. All of the participants continued with their medical therapy as prescribed by their treating physician. The participants were advised to avoid foods rich in nitrate (e.g. beetroot and green leafy vegetables such as spinach and rocket) if they were not already part of their regular diet. As mentioned before, NO can be converted non-enzymatically in the nitrite/nitrate pathway. Hence, when not part of a regular diet, consumption of such vegetables must be considered a cofounding factor. No additional nutritional advice or supplements were given to the patients. The patients

were asked to maintain their usual eating and physical activity habits throughout the study.

Endothelial Function

Brachial artery images were obtained with a high-resolution (12 MHz) linear-array vascular ultrasound scanning transducer (Vivid 7; GE Healthcare). The participant was lying in the supine position with the right arm in a comfortable position for imaging of the brachial artery. A BP cuff was placed proximal to the transducer on the forearm, and after 10 minutes of rest, the cuff was inflated to at least 200 mmHg, or 50 mmHg over the systolic pressure, for exactly five minutes. Longitudinal brachial artery images were recorded during the 30 seconds before occlusion and for 150 seconds following cuff deflation.

Oxygen uptake kinetics

VO_2 kinetics quantifies the rate of increase in VO_2 reflecting the change in muscle energetics during exercise, metabolic control and efficiency of skeletal muscle contraction (Korzeniewski and Zoladz, 2006). Following assessment of FMD of the brachial artery, the measurement of VO_2 kinetics started with a three-minute seating

rest on the cycle ergometer to obtain resting VO_2 data. Next, the participants were instructed to cycle at a rate of 70 rpm against a resistance corresponding to 30% of peak load for six minutes. After six minutes of cycling, the participants remained seated on the bike for additional six minutes, after which they performed a second six-minute bout. Resting VO_2 was given as VO_2 during the final minute before exercise. The actual achieved VO_2 during the entire exercise bout was calculated as the sum of VO_2 above resting levels. Since we were interested in examining the skeletal muscle oxidative capacity, we eliminated the first 20 seconds of data after the onset of exercise as this phase represents the early fast increase in VO_2 that is mainly attributed to the increase in cardiac output and thus, pulmonary blood flow, rather than changes in VO_2 in the muscle (Xu and Rhodes, 1999).

Statistical analysis

Statistical analyses were performed using SPSS (version 20; Chicago, Illinois, USA). All data are expressed as mean ± standard deviation (SD), unless specified otherwise. The Shapiro-Wilk test was used to check the normality of the data. Where parametric assumptions were met (data on endothelial function), paired student t-tests were used to compare the data between treatments. Otherwise (data on VO_2 kinetics), the Wilcoxon signed-ranked test was applied. Statistical significance was established at $P<0.05$ (2-tailed). Power calculations were not conducted *a priori* for this study.

Results

A percutaneous coronary intervention was performed in seven subjects and coronary arterial bypass graft in three. Medications included antihypertensive drugs (9 participants) including beta-blockers (6), antithrombotics (9), lipid-lowering (9) and glucose-lowering (2) drugs. The supplementation with RV implemented in this study was well tolerated with no adverse side effects.

Endothelial function

The average diameter of the brachial artery of the patients in our study is in agreement with a the SAINTEX-CAD sub study, in which endothelial function of this vascular bed was investigated in 200 CAD elderly subjects (Van Craenenbroeck, et al., 2015). There was no significant difference in the baseline or post-occlusion diameters of the brachial artery before and after supplementation with RV (Table 3). Further, we noted no significant difference in FMD under the two conditions (Figure 31 and Table 3).

Oxygen Kinetics

The VO_2 responses during exercise before and after the supplementation with RV are shown in Figure 32. There was no effect of RV on the average mean response time (MRTs), or the VO_2 steady-state. Likewise, oxygen deficit did not differ between placebo and RV. Median and range values are shown in table 3.

TABLE 3. EFFECTS OF RV ON THE DIAMETER OF THE BRACHIAL ARTERY OF ELDERLY CORONARY ARTERY DISEASE PATIENTS.

Values are means ± SD.

Diameter (mm)	Placebo	Resveratrol	p-value
Baseline	3.6 ± 0.7	3.7 ± 0.4	0.8
Post-occlusion	3.8 ± 0.8	3.7 ± 0.5	0.8
p-value	0.8	0.7	

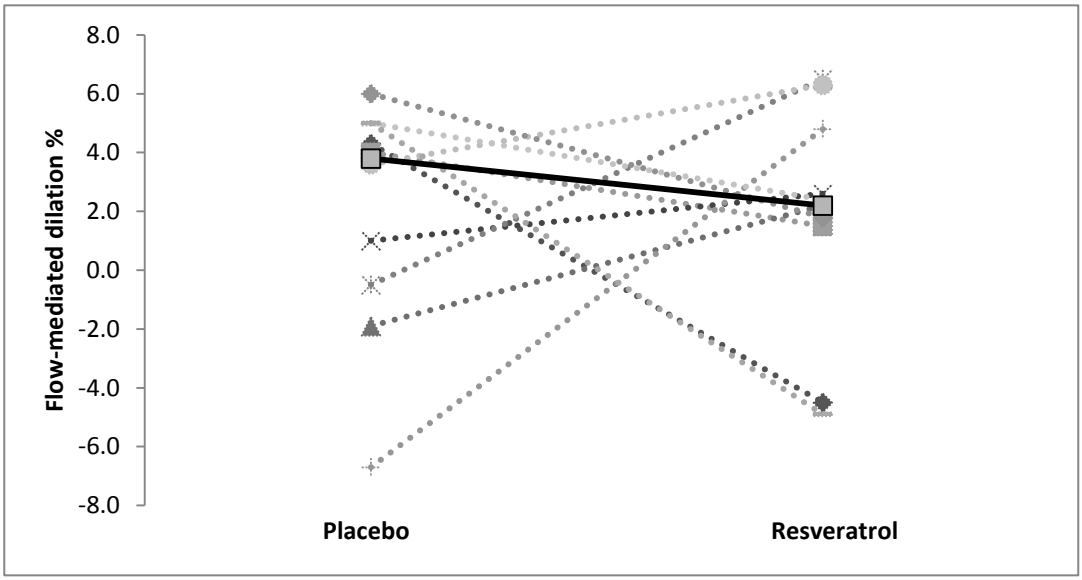

FIGURE 31. EFFECTS OF RV ON FMD OF THE BRACHIAL ARTERY.

Individual changes in percentage of flow-mediated dilation of the brachial artery of elderly coronary artery disease patients following supplementation with placebo and RV (dotted shaded lines). The group mean change is represented with the solid line. We found no difference between placebo and RV.

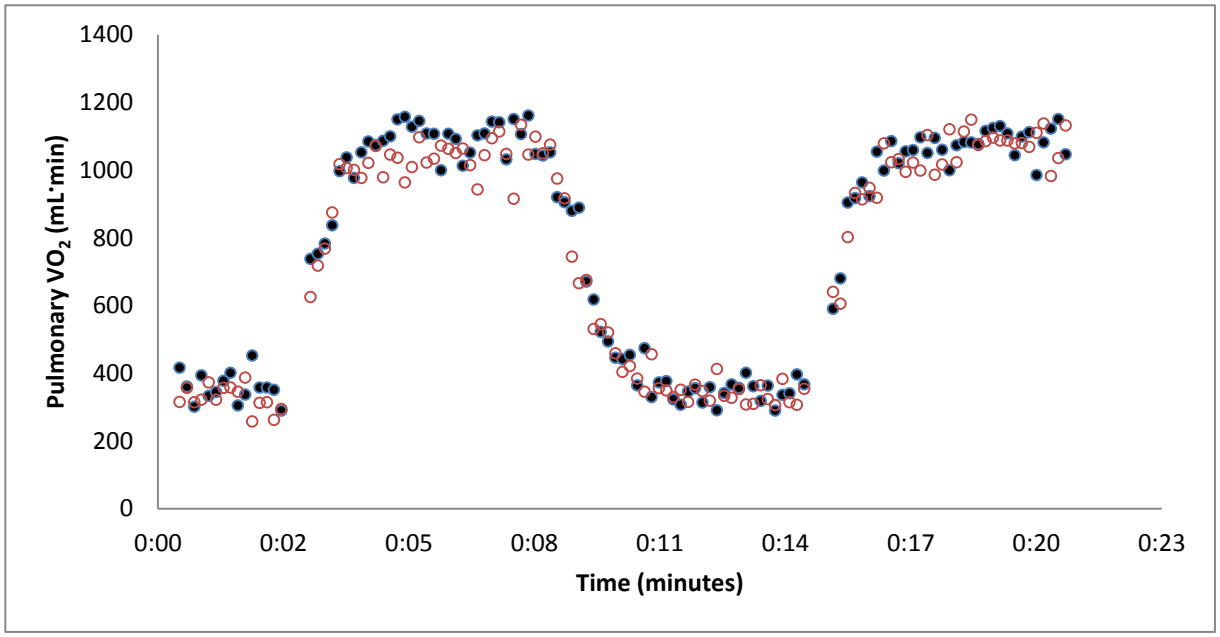

FIGURE 32. EFFECTS OF RV ON VO_2.

Group mean response in VO_2 during two bouts of exercise in elderly coronary artery disease patients following supplementation with placebo (open circles) and RV (solid circles). We found no difference between placebo and RV.

TABLE 4. EFFECTS OF ACUTE SUPPLEMENTATION WITH HIGH DOSES OF RV ON VO_2 OF ELDERLY CORONARY ARTERY DISEASE PATIENTS.

MRT: mean response time.

VO_2	Placebo		RV		p-value
	Median (range)		Median (range)		
MRT (s)	46.3	24.3-92.3	42.3	22.5-69.5	0.6
Steady-state mL·min	1109	756-1585	1101	756-1628	0.6
Oxygen Deficit mL·min	536	224-1150	662	213-1899	0.8

Discussion

The main finding of this study is that acute supplementation with high doses of RV had no effect on FMD and VO_2 kinetics in older CAD patients. In contrast to our hypothesis, RV improved neither baseline diameter, dilation of the brachial artery, nor muscle oxidative capacity during low-intensity exercise.

Dose of RV

In our study, the participants received ~1 g RV per day for three consecutive days and 330 mg on the fourth day, one hour before the physiological testing. In humans, 1 g RV per day is considered the highest oral dose without any secondary effects (Vang et al., 2011). The bioavailability of RV is low in humans due to its metabolism in the liver. The half-life of RV in plasma is approximately 9.5 hours (Walle et al., 2004). Even though absorption from orally consumed RV is at least 70%, a single dose of 25 mg leads to peak levels in plasma of RV metabolites of 2 μM and only trace amounts of non-metabolized RV (Walle et al., 2004). The dose used in our study, which was 10-fold higher than previous studies (Walle et al., 2004), should have led to approximately 80 μM of RV metabolites in plasma after the last meal of the day and to approximately 26 μM just before the evaluation of physiological parameters. The dose used in our study should be adequate as a previous study found a 23%

improvement in FMD of overweight and mildly hypertensive men after a single dose of 30 mg RV (Wong et al., 2011).

Flow-mediated dilation

During muscle contraction, perfusion can increase up to 80-fold to match the energy requirements (Buckley and Bossen, 2013). RV is thought to act through SIRT1, a protein deacetylase involved in the transcriptional regulation of cell metabolism, cell cycle, and response to damage (Ma and Li, 2015). Low SIRT1 activity has been suggested to play an important role in the development of atherosclerosis in human cardiac coronary vessels (Kao et al., 2010). In line with this, RV improved endothelium-dependent and independent relaxation and aerobic capacity via activation of SIRT1, increases in the production of NO, and the activity of both eNOS and nNOS, (Nagaoka et al., 2007; Arrick et al., 2011). In obese men, acute and chronic supplementation with RV induced a broad range of cardiovascular benefits, such as reductions in BP, lower levels of circulating markers of inflammation, glucose and intrahepatic lipid content (Timmers et al., 2011), as well as improvements in FMD (Wong, Berry, et al., 2013). As older patients with CAD present high levels of pro-inflammatory cytokines and overproduction of free radicals, which are partially responsible for the decline in cardiovascular and muscular function, we hypothesized that a three-day supplementation with high doses of RV would improve FMD, oxygen

supply to, and consequently the oxidative metabolism in the working muscle of such patients. However, our findings do not show such an effect and are in agreement with recent studies that reported no improvement in skeletal muscle function in mice (Ballak et al., 2015) or BP in humans after treatment with RV (Gliemann et al., 2013).

It is important to note that part of the controversy might be related to the dose of RV. It has been shown *in-vitro* that low doses of RV (1-10 μM) stimulate proliferation of human umbilical vein endothelial cells, the expression of vascular endothelial growth factor (Wang et al., 2010) and promoted myoblast sprouting and migration (Bosutti and Degens, 2015), whereas higher doses were detrimental (Wang et al., 2010). Conflicting findings have been published in humans. Some reports indicate that acute (up to 270 mg) and chronic (75 mg·day for six week) supplementation with RV improve FMD of the brachial artery in overweight and hypertensive, and obese adults, respectively (Wong et al., 2011; Wong, Berry, et al., 2013). On the other hand, chronic supplementation with RV (250 mg·day for eight weeks) in a group of exercising elderly men decreased the vastus lateralis interstitial levels of PGI_2 and increased the concentration of thromboxane synthase. RV also abolished the positive effects of exercise blood lipids such as low-density lipoproteins and triglycerides (Gliemann et al., 2013). Similar results have been observed in overweight elderly humans, in which RV (150 mg·day for four weeks) did not alter the profile of blood lipids, glucose, insulin or BP (van der Made et al., 2015).

Oxygen kinetics

Although several studies have been conducted to establish a relationship between nutritional supplements and changes in exercise capacity, to the best of our knowledge this is the first study exploring the effects of RV on VO_2 kinetics in CAD patients during constant low-intensity exercise. VO_2 kinetics is the result of a delicate interplay between the various mechanisms regulating O_2 delivery to and O_2 utilization by the skeletal muscle. VO_2 kinetics is significantly correlated (r=0.80) with peak VO_2 (Powers et al., 1985) and skeletal muscle oxidative capacity (r=0.33) (Coen et al., 2013). With age, absolute VO_2 levels decrease. Further, mean response times for VO_2 on-kinetics have been reported to increase linearly 0.7 $s \cdot yr^{-1}$ after the age of 30. Our findings are in agreement with published reports for older adults in which τVO_2 spans from 39 to 61 seconds (Murias and Patterson, 2015) . Previous studies in mice found that RV (0.2%; w/w for 13 weeks and 400 mg·kg·day for 15 weeks, respectively) improved exercise, and aerobic capacity via increased mitochondrial function (Lagouge et al., 2006; Murase et al., 2009). However, such benefits of RV have not been found in healthy, sedentary adults supplemented with ~1g of RV per day for one week (Voduc et al., 2014).

Study limitations

Limitations to our study include possible masking effects caused by the medication taken by the participants. As mentioned above, supplementing with RV would make clinical sense when trying to restore inflammation, oxidative stress, compromised endothelial function, amongst other conditions and some medications will have this effect. Some participants took anti-hypertensive and lipid-lowering agents that may have a significant impact on the vasodilatory capacity of arteries. Further, medication for hypercholesterolemia can influence cardiovascular parameters and skeletal muscle function (Ceriello et al., 2005). At this point, we are not able to make any statements regarding redundant effects between these drugs and RV. However, the nutraceutical potential of RV in CAD patients would only be concrete if present in addition to common prescribed medication. It is important to highlight that CAD elderly patients constitute an ideal target population for nutraceutical supplementation aimed at improving endothelial function and muscle oxidative capacity. Some have argued that populations with high levels of oxidative stress and inflammation, such as CAD elderly patients, will benefit the most from supplementation with anti-oxidants (Bast and Haenen, 2013) . Hence, different from young, healthy adults in which oxidative repair is appropriate and inflammation is not actually occurring in significant levels, there is, in theory, clinical justification to supplement CAD patients with RV. However, we acknowledge we did not determine oxidant status or circulating markers of inflammation in our study and such

physiological and biochemical aspects of the population are in our study only speculative. We also acknowledge as a limitation the lack of identification of RV metabolites in blood during supplementation and before the measurement of physiological variables. This would help draw a more detailed picture of the molecules reaching the target tissues and their biological activity *in-vivo*. As mentioned above, the participants were enrolled in a cardiac rehabilitation programme at KU Leuven. Unfortunately, ethical approval for blood sampling would have required to postpone the study for at least three months and risk a lower number of participants available.

Conclusions

In conclusion, short-term treatment with high doses of RV does not improve endothelial function or VO_2 kinetics in elderly CAD patients. Given the discrepancy in the published findings, it is possible that the promising data obtained in other experimental models thus far simply do not offer the same translational potential in humans, or at least in CAD elderly patients.

Chapter 5

Summary and General Discussion

The experiments presented in this thesis were designed to study the effects of RV on endothelial function during ageing. Considering the socio-economic burden of the increased prevalence of cardiovascular complications in old age, the development of nutraceutical strategies to ameliorate these complications has become paramount. The findings presented here on mice, cell culture and humans can be summarized as follows:

- The isolated pressurized femoral artery of 26-month old mice does not develop compromised endothelial function when compared to that of 4-month old mice, as reflected by similar levels of dilation in response to ACh (10^{-9} – 10^{-3} M) and intraluminal flow (5-10 $\mu L \cdot min^{-1}$; Chapter 2);
- The addition of inhibitors of endothelial factors LNNA, apamin + Tram 34 or indomethacin have no significant effect on ACh-induced dilation of the isolated pressurized femoral artery of both young and old mice (Chapter 2);
- Despite similar levels of FMD in the isolated pressurized femoral artery of young and old mice, there are differences in the role that endothelial factors play in dilation with ageing; addition of LNNA or apamin + Tram 34 significantly reduced dilation regardless of age. However, addition of indomethacin had no effect on dilation in the young whereas it abolished it in the old (Chapter 2);
- The isolated pressurized mouse femoral artery, regardless of age, shows an initial maximal increase in diameter followed by a sustained plateau phase in

response to intraluminal flow. In the presence of flow, the artery auto-regulates its diameter back to pre-constricted levels. Such capacity for auto-regulation is reduced upon addition of LNNA, apamin + Tram 34 or indomethacin (Chapter 2);

- In settings of pressure myography, no conversion of *trans*-RV (97% purity) to *cis*-RV, or any of its metabolites is apparent. However, a significant reduction in its concentration over time does occur (Chapter 3);
- RV causes differential effects on ACh-induced and FMD of the isolated pressurized mouse femoral artery; whereas it increases ACh-induced dilation it compromises FMD (Chapter 3);
- RV on its own and in combination with inhibitors of endothelial factors reduce the capacity of the isolated pressurized mouse femoral artery to auto-regulate diameter in the presence of intraluminal flow (Chapter 3);
- During the first 10 minutes of exposure to ACh (10^{-5} M) and intraluminal flow (8-10 $\mu L \cdot min^{-1}$), HCAEC produced significantly less NO when previously incubated with RV than under control conditions. However, when previously incubated with RV and upon exposure to ACh (10^{-5} M) for one hour, the concentration of NO increased significantly (Chapter 3);
- Acute supplementation with high doses of RV failed to improve FMD of the brachial artery or VO_2 kinetics in elderly coronary artery disease patients (Chapter 4).

The C57BL/6 mouse has become a common model of vascular ageing in biological research due to the risks and limitations of invasive studies in (elderly) humans (Lesniewski et al., 2009). We carefully designed these studies so there would be no variation in age within a given age group at the time of the experiments. Mice were either 4 or 26 months old in the young and old groups, respectively. C57BL/6 mice are considered to live up to 32 months (Turturro et al., 1999). However, at that age the survival rate is approximately 10%. Previous studies indicate that C57BL/6 mice develop compromised endothelial function in the aorta and carotid arteries from 22 months of age (Blackwell et al., 2004; Didion et al., 2006; Brown et al., 2007; Lesniewski et al., 2009). At that age, their life expectancy is reported to be between 50-75% (Turturro et al., 1999). Therefore, we considered that our age groups would be adequate to assess how RV may affect endothelial function in the isolated pressurized old femoral arteries.

Different mechanisms underlie ACh-induced and FMD. Autonomic ganglions release ACh, which upon binding to muscarinic receptors in the vascular endothelium promotes the production of NO, PGI_2 and EDHFs via changes in the intracellular concentration of Ca^{2+} (Thomsen et al., 2000). As discussed in Chapter 1, in response to ACh, NO and PGI_2, act downstream a signalling cascade that involves secondary messengers and the subsequent activation of protein kinases (Pacher et al., 2007; Majed and Khalil, 2012). On the other hand, the ACh-induced release of EDHFs leads to membrane hyperpolarization and the consequent dilation of the blood vessel

(Ohashi et al., 1999). In isolated pressurized skeletal muscle arterioles, for instance, this response is mediated by the opening of all three subtypes of Ca^{2+}- activated K^+ channels; small, intermediate and large, as demonstrated by blockade with apamin, Tram 34 and iberiotoxin, respectively (Dora, 2016). Likewise, vasodilators are released from the vascular endothelium in response to increases in shear stress. When the blood flow supplied by the artery to a tissue increases (higher shear stress), the artery dilates. To date, no clear consensus exists on the exact mechanisms that lead to FMD. It is assumed that the direct transmission of mechanical forces from the EC cytoskeleton to the VSMCs constitutes the stimulus for dilation (Markos et al., 2013). Since EC are constantly exposed to mechanical forces, they express a broad range of receptors that convert mechanical signals into intracellular chemical messages. These include junctional proteins (VE-cadherin, occludin), receptor kinases (vascular endothelial growth factor receptor 2 and others), integrins, focal adhesions, G-proteins, G-protein-coupled receptors, ion carriers and the glycocalyx (Chistiakov et al., 2016). Compelling evidence for this has been presented *in-vitro*. For instance, platelet endothelial cell adhesion molecule-1 (PECAM-1) is a protein localized in the endothelial cell-cell adhesion site (Ren et al., 2015). Physiological levels of shear stress induced the tyrosine phosphorylation of PECAM-1 in a process independent of Ca^{2+} mobilization or K^+ channel activation (White and Frangos, 2007). Once phosphorylated, PECAM-1 plays a critical role in the discrimination between prolonged steady and temporal changes in shear stress (White and Frangos, 2007), and the corresponding production of NO associated with each. Whereas prolonged steady shear stress causes a small increase in the levels of NO (Kuchan et al., 1994),

the sudden increase in laminar flow causes a significant and much larger burst in the production of NO, as observed in our study. The production of NO in these scenarios is both calcium- and G protein- independent (Kuchan et al., 1994). To date, the understanding of molecular networks associating the endothelial-mediated mechanotransduction of shear stress with the precise activation of eNOS, PGI_2 synthase or the release of EDHFs is limited.

When studied *ex*-vivo, the isolated pressurized femoral artery of 26-month-old mice shows preserved endothelial function in response to ACh and intraluminal flow. More importantly perhaps than the findings of preserved endothelial function in this vascular bed, is the fact that endothelial factors play different roles in ACh-induced and FMD. We noted no greater contribution of any particular vasodilator to ACh-induced dilation with age. Our findings add to an increasing body of evidence suggesting heterogeneity in age-related vascular responses (Støen et al., 2001; Shi et al., 2008; Puzserova et al., 2014). In response to ACh, the role that vasodilators play in vessel dilation is proportional and redundant so they guarantee maximal dilation (or at least levels of dilation not significantly different from controls), even in the presence of LNNA, apamin + Tram 34 or indomethacin. On the other hand, in response to intraluminal flow, predominant roles emerged. NO and EDHFs are pivotal for dilation regardless of age. However, inhibition of PGI_2 with indomethacin has no effect on maximal FMD in the young whereas it abolishes it in the old.

The delivery of nutrients to, and the removal of waste from the tissues are critical to maintain homeostasis and depend largely on the capacity of the vessel to dilate and constrict to match the need of the downstream tissues. We discussed in previous chapters how physiologically it would make sense to have redundant vasodilator mechanisms in place. For instance, during pathological conditions that reduce the bioavailability of NO such as hypertension and heart failure (Katz and Krum, 2001; Taddei et al., 2006; Ballard et al., 2014), such redundant systems ensure that the blood vessel could still achieve near maximal levels of dilation. The redundancy appears to be much less for FMD. As stressed before, FMD is clinically the most relevant measure between the two since it simulates more closely the *in-vivo* functioning of the vascular bed. Clearly, both approaches complement each other and important physiological insight can be gathered when applied in concert. However, considering the significance of FMD, we question the advantage of the ACh dose responses in understanding the impact of ageing on endothelial function, particularly when the femoral segments were treated with RV and completely opposite effects were observed.

There are important methodological differences in the assessment of ACh-induced dilation and FMD. Traditionally, non-pressurized vessels are preferred in vascular research because of the technical difficulties associated with pressure myography. For instance, it has been reported that only half of the vessels that dilate in response to ACh show changes in diameter in response to intraluminal flow (Liu et al., 2004).

Although the reasons for this are unknown, the authors demonstrated that increasing the extracellular concentration of ATP dramatically enhances the response to flow in non-responsive isolated vessels (Liu et al., 2004). Other methodological complications involve changes in intramural pressure associated with increases in intraluminal flow. Pressure along the vessel needs to be constantly monitored and adjusted for so increases in diameter are not the consequence of mere increases in pressure. Although small changes in pressure are expected upon the introduction of flow, we are confident the variation in diameter (or lack thereof, as in the RV *ex-vivo* study) observed in our studies reflect genuine endothelium-dependent responses to flow. We constantly monitored average pressure in the presence of flow and the increase, if any, in intramural pressure upon the introduction of flow was only approximately 1 mmHg. Moreover, any increases at the proximal end caused by the introduction of flow would be automatically compensated for by the system at the distal end. In this regard, a previous report showed that even increases in intramural pressure as high as 20-40 mmHg had no significant effect on diameter in the coronary arteries of rats (Lynch et al., 2006).

Considering the above, it becomes evident that a parallel between data gathered from agonist (ACh) and mechanical (flow) models is difficult to make. Especially from models of ACh-induced dilation, many are the assumptions made regarding the authentic functioning of the vascular bed investigated. Our findings suggest differential effects of RV on ACh-induced and FMD of the isolated pressurized mouse

femoral artery. Doses of RV >30 μM both *in-vitro* and *ex-vivo* are considered to be high (Bruder et al., 2001; Igura et al., 2001; Araim et al., 2002; Howitz et al., 2003; Yuan et al., 2010; Chen and Easton, 2011; Clark et al., 2012; Frombaum et al., 2012; Tang et al., 2012) and some have attributed the lack of beneficial effects of RV to a biphasic concentration-response relationship (Clark et al., 2012; Petit et al., 2016). Thus far, conflicting evidence has been accumulated regarding the dose-dependent effects of RV on vasodilation of isolated arteries and other cardiovascular parameters. For instance, RV (5 mg·kg·day for 14 days) improved flow-mediated outward remodelling of rat mesenteric arteries. However, at higher concentrations (37.5 mg·kg·day for 14 days), RV induced excessive contractility, increased the cross-sectional area and the wall-to-lumen ratio of the same arteries (Petit et al., 2016). On the other hand, RV has been reported to induce significant concentration-dependent dilation of isolated pre-constricted rat mesenteric arteries, in which high (35 μmol·L), but not low (5 μmol·L) concentrations led to maximal levels of dilation (>95%) (Naderali et al., 2001). Similar results have been observed in pre-constricted isolated mesenteric and uterine arteries from guinea pigs (Naderali et al., 2000), in which RV (5-70 μmol·L) induced concentration-dependent relaxation of both arteries, with the effects being 2-fold more potent on the mesenteric and the uterine arteries (Naderali et al., 2000). It is possible that the detrimental effects of RV on FMD in our studies are due to the dose of RV used. Although one might argue that improvements in FMD could have been achieved with the use of lower doses, this is unlikely as the dose we used improved ACh-induced dilation in a concentration dependent fashion. Thus, we suggest that the effects of RV seen in the present study are related to the transduction of important

mechanical signals critical to FMD, which as explained before, are bypassed in the study of ACh-induced dilation or non-pressurized vessels. Unfortunately, most of the evidence on the vasorelaxant effects of RV has been gathered from such models. Therefore, no attention has been paid to whether RV interferes with flow-mediated endothelial mechanotransduction. In our functional *ex-vivo* study, the isolated pressurized mouse femoral arteries were exposed to increasing doses of ACh or intraluminal flow for approximately 3-5 minutes per dose of ACh or flow rate. Interestingly, in our *in-vitro* study RV reduced the production of NO during the first 10 minutes of incubation with ACh. However, following incubation with ACh for one hour the production of NO increased significantly. This suggests that the improvements in ACh-induced dilation of the mouse femoral artery caused by RV might be NO-independent. Moreover, bearing in mind that in our study FMD of the isolated pressurized mouse femoral artery was largely dependent on NO, we could argue that RV negatively affects FMD via the inhibition of NO.

In response to increased oxygen demands, the blood flow entering a muscle can increase up to 80-fold (Buckley and Bossen, 2013). In light of the dependence of physical performance on muscle blood flow, we hypothesized that physical performance in coronary artery disease elderly patients would improve as the result of an enhanced endothelial-dependent dilation following acute supplementation with high doses of RV (1 g·day for three days). We found that acute and high doses of RV failed to improve FMD or VO_2 kinetics. As with the murine studies, the high dose of

RV used in our study might have blunted vascular and oxidative responses (Gliemann et al., 2013, 2014; Olesen et al., 2014). Evidence of the efficacy of acute supplementation with RV on FMD in humans is limited. A single dose of 600 mg of red wine polyphenol extract caused an absolute increase of 1.92% in FMD of the brachial artery of patients with coronary heart disease (Lekakis et al., 2005). However, the concentration of RV in this extract was low (0.54 mg). Further, RV was only one of many other polyphenols such as epicatechin (2.59 mg) and gallic acid (1.24 mg) with proven vasoactive properties and the most likely candidates to have caused such effect (Wong, Coates, et al., 2013). In overweight and mildly hypertensive adults, a single dose of *trans*-RV 270 mg caused a mean absolute increase in FMD of brachial artery of 3.67% one hour after consumption (Wong et al., 2011). On the other hand, recent evidence has demonstrated that chronic supplementation with RV inhibits, rather than activates AMP-activated protein kinase and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) in humans (Greene et al., 2012; Skrobuk et al., 2012; Yoshino et al., 2012; Gliemann et al., 2013). The activation of these proteins is critical for mitochondrial biogenesis and consequent increases in VO_2 (Gliemann et al., 2013). Further, RV has been reported to increase the protein content of thromboxane synthase in the vastus lateralis muscle of elderly humans following daily supplementation with 250 mg for eight weeks (Gliemann et al., 2013). Since thromboxane synthase and PGI_2 synthase compete for substrates in the arachidonic acid pathway, the authors explained the lack of positive effects on BP on the shift towards vasoconstrictor mechanisms. It is worth mentioning at this point that in addition to its putative cardiovascular properties RV is also an antioxidant. RV

can scavenge $O^{2}\bullet^{-}$ and the hydroxyl radical (•OH) (Leonard et al., 2003; Cao and Li, 2004). It has been well demonstrated that ROS serve a broad range of physiological functions including vasodilation and oxygen consumption, delivery and utilization (Barja, 2007; Radak et al., 2008; Zhang et al., 2012). Consequently, one could argue that by supplementing with high doses of RV and increasing the antioxidant defence, the signalling function of ROS in the endothelium and the skeletal muscle might have been negated (Gliemann et al., 2013). In support of this, previous studies have shown that in young men, daily supplementation with vitamin C (1000 g) and vitamin E (400 UI) for four weeks abolished the exercise-induced improvements in insulin sensitivity, expression of PGC1α and antioxidant enzymes (Ristow et al., 2009). Further, daily use of statins (inhibitors of NADPH oxidase; 40 mg) by overweight adults for 12 weeks significantly blunted the exercise-induced increases in $VO_{2\ peak}$ and citrate synthase (Mikus et al., 2013).

Future directions

The evaluation of vascular responses *ex-vivo* with pressure myography provides a well-controlled model for the understanding of the physiopathological properties of blood vessels. However, there are important limitations to this approach. The cross-talk between different molecules in blood, such as growth factors, hormones neurotransmitters and cytokines with the endothelium, for instance, is difficult to reproduce. Further, the communication between the adventitia and surrounding

tissue, such as skeletal muscle, is absent in this preparation (Lu and Kassab, 2011). The contraction of skeletal muscle, as discussed previously, is critical for the dilation and constriction of arteries and arterioles. Clearly, the data gathered from this model is representative of the general functioning of the blood vessel, but by no means is it conclusive (Lu and Kassab, 2011). Likewise, the risks associated with the administration of vasodilator inhibitors in the elderly and the consequent lack of characterization of the dilator response in humans make the interpretation of our results limited. Considering the clinical value of FMD, it is surprising that there is currently no generally accepted procedure in rodents that is in line with that in humans. Some have proposed the use of Doppler ultrasound, as routinely used in humans, in the femoral artery of both rats and mice with promising results (Heiss et al., 2008; Schuler et al., 2014). Critical issues such as dose and duration of treatment with RV, as well as the identification of the metabolite reaching the target issue still need to be thoroughly addressed before the translational potential of RV to improve endothelial function is confirmed. Evidence attributing positive effects of RV to FMD in both humans and other animals is fairly recent and no enough data have been gathered in this area (Wong, Coates, et al., 2013). In view of the opposite effects of RV on ACh-induced and FMD, we believe our findings point to important new avenues of research, particularly the understanding of the effects of RV on flow-mediated endothelial mechanotransduction. The application of laminar flow to cultured EC, as used in our study, provides a useful tool for the investigation of casual relationships between shear stress and endothelial function. As mentioned above, a large family of membrane associated proteins have been suggested to mediate the transduction of

mechanical stimuli within the blood vessel, which include PECAM-1, ion channels, receptor-tyrosine kinases, adhesion molecules and the glycocalyx (Kwak et al., 2014). Interestingly, for some of these mechanoreceptors pleiotropic properties have been described. PECAM-1 on bone marrow cells, for instance, is pro-atherogenic irrespective of the hemodynamic environment whereas on EC is anti-atherogenic in high shear environments (Harrison et al., 2013). This suggests that targeting this receptor would require a cell-type and context-specific strategy (Harrison et al., 2013). PECAM-1, as any other mechanoreceptor might respond in similar fashion and depending on the vascular bed localized, to different RV metabolites, doses of RV or duration of treatment. It remains for future research to continue with this approach and draw a more detailed picture of the effects of RV on flow-mediated endothelial mechanotransduction.

Conclusions

Taken together our findings indicate that despite similar levels of maximal dilation of the isolated pressurized mouse femoral artery in response to intraluminal flow during ageing, important changes in the predominance of vasodilators occur, particularly PGI_2. RV induced opposite effects on lumen diameter of isolated pressurized the mouse femoral artery that depended on the dilator stimulus (ACh *vs.* intraluminal flow). RV decreased the production of NO in EC in response to ACh and laminar flow during the first 10 minutes of exposure to either stimulus. However,

when cells were exposed to ACh for one hour, the concentration of NO increased significantly. Considering that in our study, FMD of isolated pressurized mouse femoral artery was largely dependent on NO, such decrease in the production of NO in response to laminar flow could be attributed to the negative effects of RV on FMD. In partial agreement with the animal studies, RV failed to improve endothelium-dependent dilation of the brachial artery and VO_2 kinetics in coronary artery disease elderly patients. The mechanisms underlying these responses might be associated with compromised flow-mediated endothelial mechanotransduction and/or biphasic concentration-responses of RV.

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Appendix 1

Supplementary Data

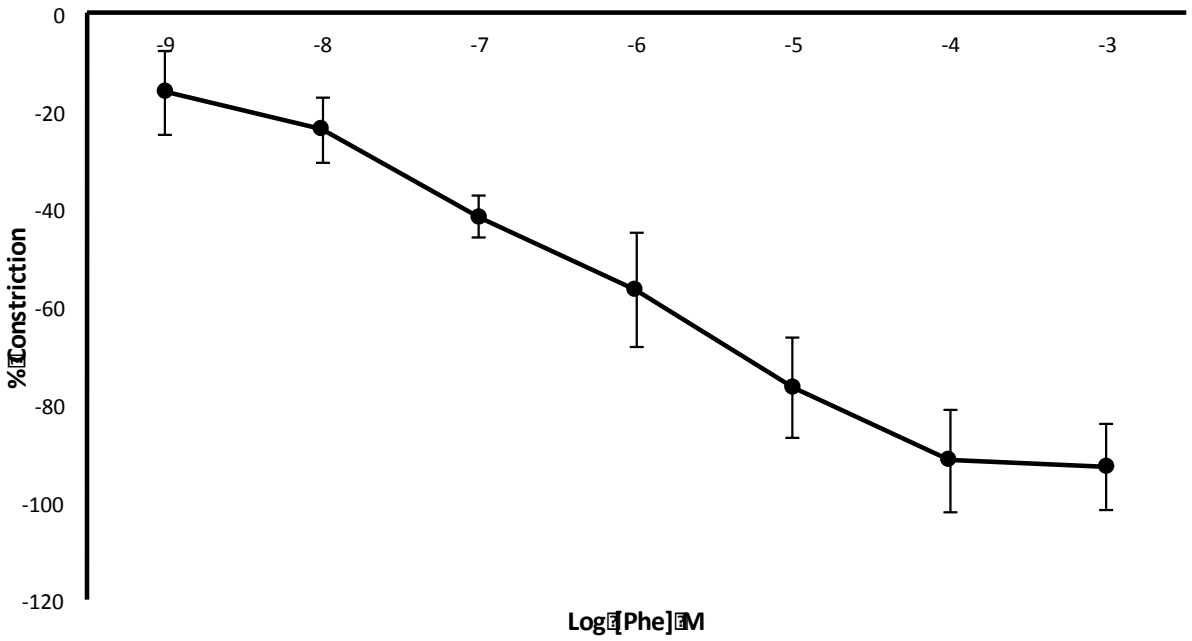

FIGURE 33 PHENYLEPHRINE (PHE) DOSE-RESPONSES

Constrictor responses of the isolated pressurized femoral artery from 4-month old mice (n = 5) to increasing doses of Phe (10^{-9} – 10^{-3} M). Optimal levels of constriction (~80%) were achieved with Phe 10^{-5} M. Data are means ± SEM.

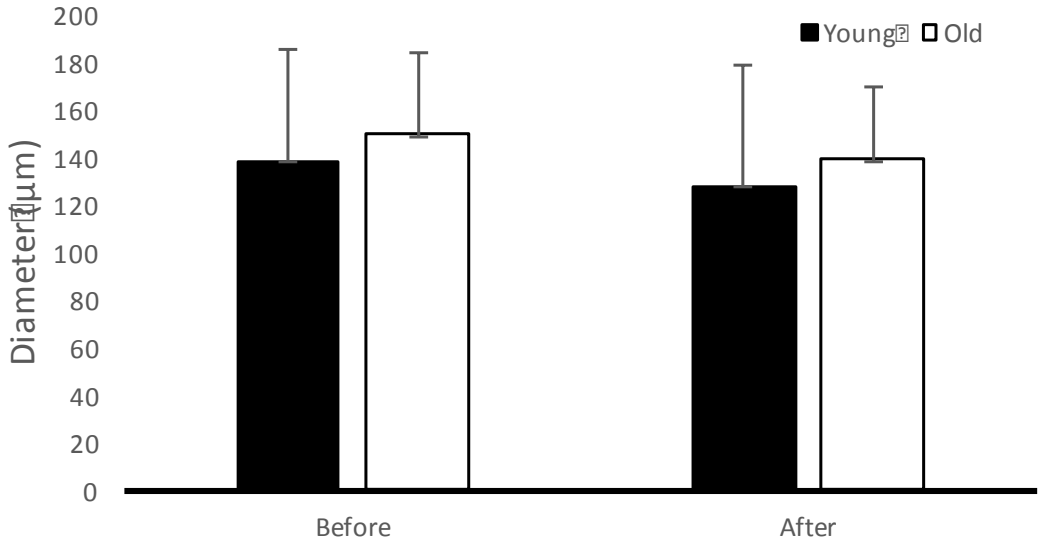

FIGURE 34 RESTING DIAMETERS BEFORE AND AFTER EQUILIBRATION.

Resting diameters of the isolated pressurized femoral artery from 4-(young; *n* = 11) and 26-month old mice (old; *n* = 6) before and after an equilibration period of one hour in PSS and intraluminal pressure of 60 mmHg. We observed no development of myogenic tone in either group. Data are means ± SEM.

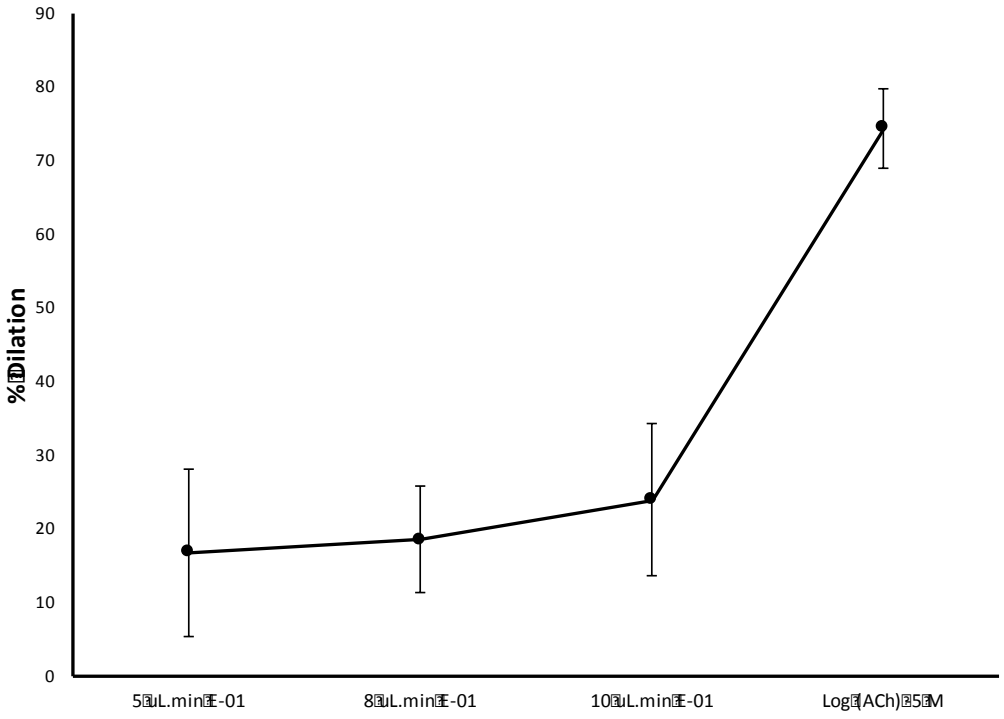

FIGURE 35. ENDOTHELIAL INTEGRITY AFTER THE INTRODUCTION OF INTRALUMINAL FLOW.

ACh (10^{-5} M) induced dilator responses are preserved in the Phe (10^{-5} M)- pre-constricted isolated pressurized femoral artery from 4-month old mice (*n* = 5) after introduction of intraluminal flow (5-10 $\mu L \cdot min^{-1}$). Intraluminal flow was introduced for 4-5 minutes per flow rate with a period of two minutes with no flow in between. Data are means ± SEM.

Appendix 2

Conferences, talks and lectures

The following are the scientific meetings, talks and lectures in which I presented the findings that comprise this thesis:

- Differential effects of resveratrol on acetylcholine-induced and flow-mediated dilation of the mouse femoral artery (**poster presentation**). British Cardiovascular Society Annual Conference, Manchester, UK, June 2016;
- The vascular effects of resveratrol on the isolated mouse aged femoral artery (**lecture**). Biomedical Cell Biology Unit, Manchester Metropolitan University, Manchester, UK, Jan 2016;
- The role of antioxidants in improving vascular function (**conference**). VIII MMU Postgraduate Research Conference, Manchester Metropolitan University, Manchester, UK, Nov 2015;
- Impaired mobility in aging; the role of antioxidants in improving vascular and muscular function (**conference**). V MOVE-AGE Annual Conference. Manchester, UK, Sept 2015;
- The vascular effects of resveratrol on aged mouse femoral arteries (**poster presentation**). British Cardiovascular Society Annual Conference, Manchester, UK, June 2015;
- Acute supplementation with resveratrol does not improve flow-mediated dilation in coronary arterial disease elderly patients (**poster presentation**). European College of Sport Science Annual Meeting, Malmo, Sweden, June 2015;

- British Heart Foundation Open Day (**public engagement**). Manchester Art Gallery, June 2015;
- Supplementation with resveratrol does not affect muscle oxidative capacity (**poster presentation**). EuroPrevent Congress, Lisbon, Portugal, May 2015;
- Impaired mobility in aging; the role of antioxidants in improving vascular and muscular function (**conference**). IV MOVE-AGE Annual Conference, Amsterdam, The Netherlands, July 2014.

Appendix 3

Publications

The Effects of Resveratrol on Aging Vessels[ℜ]

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Abstract

Aging is a major risk factor for the development of cardiovascular disease. Despite a significant reduction in the mortality and morbidity rates over the last decade, the socio-economic burden of cardiovascular disease is still substantial. Consequently, there is a considerable need for alternative strategies, such as nutraceutical supplementation, that delay the functional vascular decline present in the elderly. Compromised autophagy and oxidative stress (OS) are considered major causes of the age-related endothelial dysfunction. OS reduces the bioavailability of nitric oxide (NO), which has been associated with hypertension, arteriosclerosis and a reduced vasodilatory response. High levels of free radicals and the low bioavailability of NO lead to a positive feedback loop of further OS, organelle damage, poor repair and endothelial dysfunction. Here we draw attention to the relationship between OS and autophagy in the aged vasculature. We have reviewed the published literature and provided arguments that support that treatment with resveratrol stimulates autophagy and thereby has the potential to restore oxidative balance in the endothelium, which indicates that treatment with resveratrol might have therapeutic potential to restore endothelial function in the elderly.

Keywords: Antioxidant; Autophagy; Endothelium; Oxidative Stress.

1. Introduction

Advances in medicine have allowed humans to become older. Life expectancy in developed countries is now on average 70 years and it is estimated that by 2030 20% of the world's population will be older than 65 years (North and Sinclair, 2012). However, aging is associated with a steady and rapid physiological decline. Aging, for instance, is a major risk factor for the development of cardiovascular disease. In 2014, cardiovascular disease was the second most common cause of death in the UK with approximately 155,000 deaths (Cardiovascular Disease Statistics 2015). Despite a significant reduction in the mortality and morbidity rates over the last decade, the socio-economic burden of cardiovascular disease is still substantial (Bhatnagar et al., 2015). Consequently, there is a considerable need for alternative strategies such as nutraceutical supplementation that delay the functional decline present in old age.

The aged vasculature is characterized by increased arterial thickness, stiffness and endothelial dysfunction. Such structural and functional changes result in hypertension, arteriosclerosis, stroke, poor tissue perfusion, among other pathological conditions (North and Sinclair, 2012). At the molecular level, aging is characterized by a reduced bioavailability of nitric oxide (NO), increased production of collagen by vascular smooth muscle cells, and changes in the expression of proteins that regulate calcium handling (Strait and Lakatta, 2012). NO plays a pivotal role in endothelium-dependent dilation. Further, NO is critical for the prevention of

thrombosis and inhibition of platelet aggregation (Tousoulis et al., 2006; Vora et al., 1997). Hence, a reduced bioavailability of NO is associated with endothelial dysfunction. One of the most important factors affecting NO synthesis in the aged vasculature is oxidative stress (OS). Within the endothelial cell, cumulative oxidative damage leads to organelle dysfunction, and compromised repair or renewal (Irani, 2000).

In this regard, resveratrol (RV) has received considerable attention during the last decade. RV is a naturally occurring polyphenol found mostly in the skin of red grapes, peanuts, and blackberries (Li et al., 2012). Flow-mediated dilation (FMD) refers to the changes in vessel diameter caused by increases in shear stress. FMD is considered a surrogate of endothelial function and in humans is measured by ultrasound in superficial arteries such as the brachial and femoral arteries (Currie et al., 2014). A growing body of evidence suggests that RV improves FMD (Wong et al., 2013), reduces blood pressure (BP), the circulating levels of inflammatory molecules (Timmers et al., 2011), and myocardial damage during ischemia-reperfusion (Bradamante et al., 2004). The mechanisms by which RV confers vascular protection involve a higher biosynthesis of NO, increases in the activity of proteins that regulate oxidative metabolism, increases in the activity several antioxidant enzymes, as well as the stimulation of cellular self-repair processes (collectively known as autophagy) within the endothelial cell. Consequently, RV has become an attractive candidate in nutraceutical strategies that aim to improve vascular function in the elderly

Here we review how cumulative oxidative damage and impaired autophagy in old age might contribute to endothelial dysfunction. We discuss the potential of RV to protect against oxidative assault, promote autophagy and restore endothelial function in old vessels.

2. The vascular endothelium

The endothelium is a monolayer of cells that lines blood vessels and is the only cellular layer that separates the blood from surrounding tissue in capillaries. It acts as a permeable barrier to solutes and macromolecules, and is critical not only in the regulation of vessel diameter, but also in coagulation, angiogenesis and inflammation. Endothelial cells modulate vascular tone by releasing vasoactive molecules, such as NO (Furchgott and Vanhoutte, 1989). NO activates cGMP-dependent protein kinase, which induces vasodilation and prevents platelet aggregation (McHugh and Cheek, 1998). Furthermore, NO enhances oxidative metabolism and oxygen consumption (Clementi et al., 1999; Parihar et al., 2008), and protects against ischemia-reperfusion injury and oxidative damage (Crouser, 2004; Gourine et al., 2002; Rakhit et al., 2001).

Under normal conditions, the vascular endothelium regulates arterial tone and blood flow to match the needs of the tissue. Vascular dysfunction is evident when either the release of, or the response to vasodilators is blunted. For instance, overproduction of vasoconstrictors with a reduced bioavailability of relaxing factors, such as NO, will

lead to an increased vascular tone (Lerman and Burnett, 1992). During aging, the higher secretion of endothelial cell-derived pro-inflammatory and thrombogenic factors eventually results in irregular vasoreactivity and partial loss of endothelial function (Verma and Anderson, 2002), which underlies the increased prevalence of cardiovascular problems in old age.

There is evidence that OS and mitochondrial dysfunction play a significant role in the age-related onset of endothelial dysfunction (Brandes et al., 2005). OS will over time result in an accumulation of molecules modified by reactive oxygen and nitrogen species, which leads to compromised organelle and ultimately cell and tissue function. One of the mechanisms whereby the cell protects itself from the accumulation of oxidatively modified protein and dysfunctional organelles is autophagy. During autophagy, pathogens, dysfunctional organelles and harmful cytoplasmic constituents are sequestered into vesicles, autophagosomes, and fused with the lysosome for degradation and recycling (Kroemer et al., 2010).

3. Oxidative stress, autophagy and the aged vasculature

The capacity of the vessel to dilate in response to increased flow or vasodilating agents diminishes with age (Brandes et al., 2005). Part of the problem is related to a disturbed regulation of release of vasodilatory and vasoconstricting factors from the

endothelial cells. Many factors contribute to age-related endothelial dysfunction. Among those are decreased circulatory levels of growth factors and vasodilators, increased circulatory levels of vasoconstrictors and inflammation (Csiszar et al., 2004; Daiber et al., 2016). Today, it is widely accepted that OS plays a pivotal role in age-related endothelial dysfunction (Brandes et al., 2005). Scavenging of free radicals is necessary for the appropriate functioning of the endothelium. With age, increased production of the anion superoxide (O2•-) has been suggested to lead to a reduced bioavailability of NO, as O2•- will scavenge NO to produce peroxynitrite (ONOO−) (Blackwell et al., 2004; Ferrer et al., 2003; Hamilton et al., 2001; Rodríguez-Mañas et al., 2009; van der Loo et al., 2000). The main sources of endothelium-derived O2•- appear to be the mitochondrion, NADPH oxidase and endothelial nitric oxide synthase (eNOS) itself (Hamilton et al., 2001). Tetrahydrobiopterin (BH_4) is an essential cofactor for the NO synthases. In high concentrations, ONOO− oxidizes BH_4. In the absence of BH_4, the NO synthases become uncoupled and produce O2•-, which leads to further production of ONOO− and OS (Golbidi and Laher, 2013).

As mentioned above, aging is a major risk factor for the development of cardiovascular disease. The molecular and morphological profiles of the aged endothelial cell correspond to a state of disturbed homeostasis and poor repair (LaRocca et al., 2013). Considering that autophagy is reduced during aging and the close association between autophagy and OS, there is reason to believe that the inability to maintain autophagy is instrumental, if not critical, for the OS-induced

endothelial dysfunction in old age (Lee et al., 2012). Autophagy is involved in the expression of eNOS under steady laminar shear stress (Guo et al., 2014). Endothelial cells with knockdown of autophagy related protein (Atg) 3 display significantly lower levels of eNOS phosphorylation and are not able to produce NO in response to shear stress. This is accompanied by higher levels of reactive oxygen species (ROS) and inflammation as indicated by the increase in monocyte chemoattractant protein-1 and interleukin 8 (IL-8) (Bharath et al., 2014). The reduced bioavailability of NO and subsequent compromise of endothelial function has been associated with pathological conditions such as hypertension and atherosclerosis (Eren et al., 2013; Münzel et al., 2008).

Significant build-up of oxidative by-products results in endothelial cell death. However, such accumulation is largely prevented by autophagy. 4-hydroxynonenal (4-HNE) and acrolein, products of lipid peroxidation, have been shown to strongly induce autophagy in endothelial cells from Sprague–Dawley rat aortic explants. Further, failure to remove aldehyde-modified proteins by inhibiting autophagy accelerates aging and cell death (Hill et al., 2008; LaRocca et al., 2012). The importance of autophagy is indicated in these studies by a robust increase of microtubule-associated protein 1 light chain 3 II (LC3II) and significant vacuolization, formation of pinocytic bodies, crescent-shaped phagophores and multilamellar vesicles, all morphological characteristics of autophagy. (Hill et al., 2008; LaRocca et

al., 2012). Thus, it is possible that the inability to maintain autophagy is responsible for the OS-induced endothelial dysfunction in old age.

Recently, LaRocca et al (2012), demonstrated in a compelling study that the decline in endothelial function in elderly subjects depends on NO availability. Further, that endothelial dysfunction is associated with higher circulating levels of oxidized low-density lipoprotein, IL-6 and C-reactive protein, and a reduced expression of beclin-1, a critical protein in the initiation of autophagy, in endothelial cells isolated from the brachial artery of these subjects. Similar to their findings in humans, their experiments on the aorta and carotid arteries from aged C57BL/6 mice revealed significantly lower endothelium-dependent dilation in response to acetylcholine when compared to young controls. As expected, this was consistent with a reduced expression of eNOS, beclin 1, LC3II, WD repeat domain phosphoinositide-interacting protein 1 (WIPI-1; a key mediator of autophagy), and markedly greater levels of O2•. Interestingly, when the mice were supplemented, or the cultured cells treated with trehalose, a natural disaccharide that promotes autophagy, endothelial-dependent dilation and all markers of autophagy and OS were restored to the levels of controls. In a similar study, they proceeded to test the effects of spermidine, a natural polyamine that strongly induces autophagy (LaRocca et al., 2013). As hypothesized, treatment with spermidine enhanced autophagy, reversed elastic stiffening of the aorta, restored endothelial-dependent dilation and reduced OS in old mice (LaRocca

et al., 2013). Collectively, their findings provide strong evidence on the role that OS and impaired organelle turnover play in endothelial dysfunction during aging.

In addition to the increased production of free radicals, substantial evidence suggests that the reduction in antioxidant enzymes is also responsible for higher levels of OS in old age. In the aorta and femoral artery of rats, aging has been associated with a decrease in the activity of superoxide dismutase (SOD) and increased nitration of manganese superoxide dismutase (MnSOD) (Barton et al., 1997; Durrant et al., 2009; van der Loo et al., 2000; Zanetti et al., 2010). Evidence that the decline in antioxidant enzymes and a higher production of free radicals are associated with aging comes from MnSOD knockout models in which the animals suffered from age-dependent OS, endothelial dysfunction and cardiomyopathy (Ohashi et al., 2006; Roos et al., 2013; Strassburger et al., 2005; Wenzel et al., 2008). Further, treatment with the SOD mimetic Tempol restored endothelium-dependent dilation, and prevented the formation of atheroma plaques (Cannizzo et al., 2014; Lesniewski et al., 2009; Tatchum-Talom and Martin, 2004). Taken together, these data corroborate the effect of free radicals, particularly O2•- on the age-related endothelial dysfunction in humans and other animals.

4. The use of antioxidants

Considering the deleterious effects that cumulative oxidative damage causes in the vasculature, significant attention has been given during the last decade to the potential of antioxidants to restore oxidative balance (Conti et al., 2016). The role that free radicals play on such a broad spectrum of physiological functions has fueled the passionate debate on the potential beneficial effects of antioxidant supplementation. As elegantly explained by Bast and Haenen (Bast and Haenen, 2013), the expectations for antioxidants have been so high that the results so far have been somewhat confusing and disappointing. As mentioned above, RV has received extensive attention in this regard. As an antioxidant, RV scavenges O2•- and the hydroxyl radical (•OH), increases the activity of several antioxidant enzymes and protects lipid membranes and DNA from peroxidation and strand-breaks, respectively (Cao and Li, 2004; Leonard et al., 2003). In addition to its antioxidant effects, RV increases the activity of eNOS (Wallerath et al., 2002) and confers anti-inflammatory protection (Ungvari et al., 2009), which makes it an attractive candidate to reverse the age-related structural and functional changes in the blood vessel.

5. Considerations for the use of resveratrol

Thus far, most studies have focused on sirtuin 1 (SIRT1) as the key target of RV. Sirtuins are NAD^+-dependent protein deacetylases that regulate oxidative metabolism. SIRT1 in particular is localized in the nucleus and has been shown to increase the activity of catalase and prevent apoptosis in human endothelial cells (Alcendor et al., 2007; Li et al., 2015). However, a growing body of evidence indicates that other important signaling pathways convey the antioxidant and anti–inflammatory properties of RV. Such pathways include the activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) via AMP-activated protein kinase (AMPK) (Baur et al., 2006; Lagouge et al., 2006; Olesen et al., 2014a; Ungvari et al., 2009), as well as the down-regulation nuclear factor κB (NF-κB), which signals pro-inflammatory responses in aging (Ungvari et al., 2009, 2007). In addition, the induction of the transcription factor erythroid 2-related factor 2 (Nrf2) is responsible for some of the antioxidant and anti-inflammatory effects of RV on the endothelial cells. Upon activation, Nfr2 induces the expression of genes coding for important enzymes involved in antioxidant processes such as NADPH:quinone oxidoreductase 1, heme oxygenase-1, and γ-glutamylcysteine synthetase (Ungvari et al., 2010). On the other hand, downregulation of Nfr2 prevents the RV-induced protection from OS in human coronary arteries and impairs the dilation in response to acetylcholine in the arterioles in the gracilis muscle of aged mice (Ungvari et al., 2010).

Some of the initial enthusiasm for the nutraceutical potential of antioxidants has faded. This, mostly because we now understand that ROS serve a broad range of physiological functions. Under controlled balance free radicals regulate homeostasis and physiological angiogenesis (Bir et al., 2012). Furthermore, hydrogen peroxide (H_20_2) stimulates cell proliferation (Simon and Stutzin, 2008), promotes the secretion of growth factors (Chua et al., 1998; Colavitti et al., 2002; González-Pacheco et al., 2006), regulates apoptosis (Chen et al., 2004) and acts as a vasodilating agent in human coronary arterioles (Zhang et al., 2012). Recent evidence suggests that the H_20_2-induced dimerization of cGMP-dependent protein kinase (PKG)-Iα leads to the opening of calcium-activated potassium channels and further dilation of the vessel (Zhang et al., 2012). Hence, the paradigm that 'the more antioxidants the better' is now changing to a complementary strategy aimed to normalize the production of free radicals (Bast and Haenen, 2013). Regarding the effects of RV on the vascular endothelium in old age, conflicting data between humans and other species have been published. For instance, RV has been shown to reverse oxidative damage by reducing the expression of NADPH oxidase while increasing the expression of SIRT1 in the aorta of aged rats (Tang et al., 2012). In the cardiovascular system, the reduced expression of SIRT1 is specifically limited to aged and/or atherosclerotic vessels (Kao et al., 2010). Also, it has been demonstrated that RV prevents free radical-induced senescence in human umbilical vein endothelial cells (HUVECs) but fails to protect from oxidative damage when the gene for SIRT1 is silenced (Kao et al., 2010).

Some of the benefits of RV are mediated by stimulation of autophagy. Treatment of HUVECs with RV up-regulated the expression of sequestosome 1 (SQSMT1), an autophagosome cargo protein that selectively targets proteins for autophagy, eNOS, SIRT1, and several genes related to autophagy including *LC3B* and *AGT3* (Chen et al., 2013; Takizawa et al., 2013). In support of such protective effects, RV has also been shown to preserve telomere length and increase telomerase activity in aortic rings from aged rats (da Luz et al., 2012a). Of particular interest is the fact that endothelium-dependent dilation improved significantly after treatment with RV for six months (da Luz et al., 2012b). Recently, a causal relationship between the levels of the mammalian target of rapamycin (mTOR) and the ribosomal protein S6 kinase beta-1 (S6K1), and vascular aging was reported. Over activation of this pathway leads to aging-related disorders including cardiovascular disease (Stanfel et al., 2009). The activity of S6K1 is higher in aortic rings of old rats and in cultured senescent human endothelial cells (Rajapakse et al., 2011). Related to this, there is a higher production of free radicals and a concomitant reduced synthesis of NO. Treatment of the cells for one hour with RV restored excess production of mitochondrial O2•- and enhanced the synthesis of NO in response to acetylcholine (Rajapakse et al., 2011).

Recent studies in humans, by contrast, suggest that in aged, but healthy individuals RV blunts the positive cardiovascular effects of physical exercise (Gliemann et al., 2014, 2013; Olesen et al., 2014b). When submitted to eight weeks of high intensity training and a daily intake of either 250 mg of RV or placebo, the subjects that

received RV showed no increase in the capillary-to-fiber ratio or the concentration of vascular endothelial growth factor in the vastus lateralis. Further, supplementation with RV inhibited the exercise-induced reduction in BP and blood lipids and attenuated the gains in maximal oxygen consumption observed in the group that exercised under the placebo. Likewise, no changes were reported in markers of metabolic and inflammation status such as 3-hydroxyacyl-CoA dehydrogenase, cytochrome c oxidase I, PCG-1α, SIRT1, and TNFα. Finally, in HUVECs, RV inhibited cell migration and capillary tube formation by enhancing the nuclear translocation of the transcription factors forkhead box O (FOXO)1, FOXO3a and FOXO4 (Srivastava et al., 2010). Clearly, these results cast some doubt on the current paradigm that RV mimics the metabolic and anti-inflammatory effects of exercise and caloric restriction that promote cardiovascular health. The studies in humans, particularly in the elderly, are still only a handful when compared to those in rodents. However, as will be discussed below they call for caution when attributing to RV, or any anti-oxidant for that matter, exceptional therapeutic properties. Table 1 summarizes the studies on the effects of RV on the blood vessel. Figure 1 shows some of the mechanisms by which RV confers vascular protection.

6. Perspective

Even though some might deem the therapeutic potential of RV to be overstated, we believe that the data published thus far reflect clinical promise. Healthy populations

might not experience positive metabolic or cardiovascular effects after treatment with RV. Yet, there is no clinical justification to treat those subjects or expect such effects. Moreover, it is not clear how positive effects in an already healthy population should be interpreted (Smoliga et al., 2013). On the other hand, enough evidence has been gathered to suggest that compromised populations such as the elderly do benefit from treatment with RV (Smoliga et al., 2013). Even though a recent series of publications in aged humans (Gliemann et al., 2014, 2013; Olesen et al., 2014b) reveals no benefit from RV supplementation in a broad range of cardiovascular and metabolic parameters, further research needs to be conducted to definitely validate the claims both in favor and against RV.

We have discussed here how RV scavenges free radicals, increases antioxidant activity, and promotes autophagy and the synthesis of proteins central to oxidative metabolism in aged vessels. We have also highlighted the fact that free radicals serve as signaling molecules and as such, a controlled production is necessary for adequate functioning of the blood vessel. Interestingly, supplementation with thiol-based and other antioxidants such as tocopherol prevents ROS-induced autophagy (Underwood et al., 2010). With that in mind, some have questioned whether a higher production of free radicals during aging is in fact detrimental to cardiovascular health (Gliemann et al., 2013). Some evidence suggests that in mammalian cultured cells NO, a free radical, inhibits autophagy by nitrosylation of c-Jun N-terminal kinase 1. This ultimately leads to the disruption of the Beclin/1–hVps34 complex, which is

necessary during autophagosome formation (Sarkar et al., 2011). At first, these findings might seem in direct contrast to some of the reports discussed above. However, it is worth mentioning at this point that the production of free radicals, degradation of proteins and organelles via autophagy and the synthesis of antioxidants enzymes can be either beneficial or detrimental depending on the energy and redox status of the cell. Different oxidative modifications are particular to different types of free radicals in the same way that different antioxidants scavenge different ROS. For instance, during nutrient deprivation the cell promotes an oxidative environment by expelling glutathione (Desideri et al., 2012; Filomeni et al., 2015). Oxidized proteins become then a target of autophagy so amino acids can enter the tricarboxylic acid cycle to be used to synthesize ATP (Desideri et al., 2012; Filomeni et al., 2015). Hence, supplementation with antioxidants needs to be carefully designed. The elderly constitute that *rancid* population with high levels of inflammation and oxidative damage to which Bast and Haenen referred (Bast and Haenen, 2013). In the elderly, and in the appropriate dose, RV might *enhance* endothelial function when used as a secondary strategy to a balanced diet and regular exercise and thus, contribute to a healthier yet unavoidable, aging.

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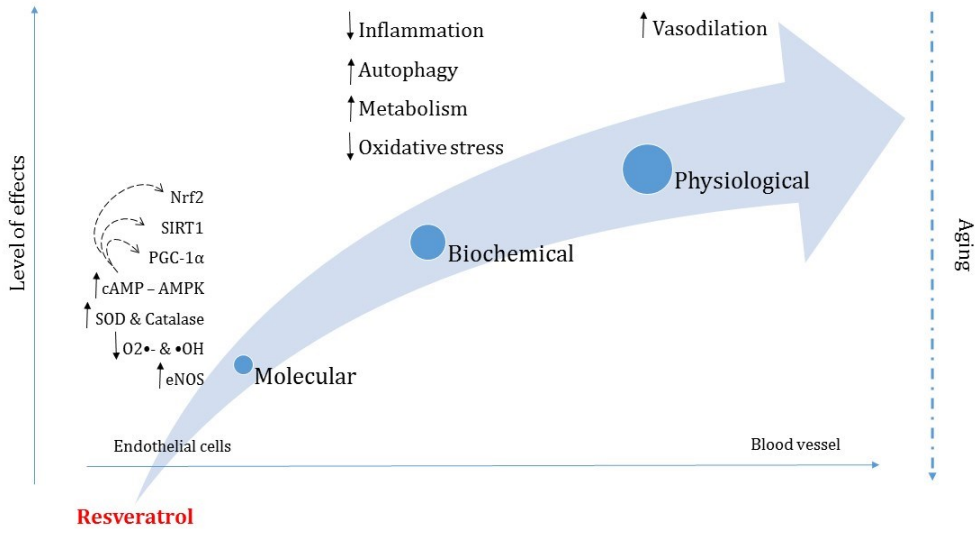

Figure 1: Schematic representation of the molecular and physiological effects of RV on the blood vessel and skeletal muscle. RV activates PCG1-α, SIRT1 and Nfr2 through the cAMP-AMPK signaling pathway. Improvements in vasodilation and attenuation of muscle mass loss are the consequence of enhanced substrate oxidation and organelle quality control together with a reduction in ROS and pro-inflammatory molecules. During aging, there is a natural decline in the capacity of the cell to renew cellular components, which results in oxidative stress, inflammation, and eventually, endothelial dysfunction and sarcopenia.

Table 1. Effects of resveratrol on the blood vessel.

Experimental Model	Cells / Tissue	Dose and Duration of Treatment	Effects	Reference
in vivo studies				
Male Fisher 344 rats	Aorta	10 mg·kg^{-1}·day^{-1} for 1 week	/NF-κB, /monocyte adhesiveness ↓ inflammatory gene expression.	Ungvari et al., 2007
Wistar rats on HFS	Aorta	50 and 100 mg/kg bw for 14 weeks	↓ gains in BW ↓ SA-β-gal-positive cells ↓ HFS-induced increase in ROS ↓ HFS-induced down-regulation of SIRT1	Tang et al., 2012
Male Wistar rats	Aorta	0.0015 mg/kg & 4 mg/kg of chow for 6 months	↑ endothelium-dependent dilation ↑ telomere length and telomerase activity ↓ expression of p53 and p16	da Luz et al., 2012a
Male Wistar Kyoto rats	Aorta	10 μmol/L for 1 hour	↑ NO production ↑ endothelium-dependent dilation ↓ activation of Akt and S6K1 ↓ production of O2•-	Rajapakse et al., 2011
Male ICR wild-type mice (Nrf2$^{+/+}$)	Arterioles in the gracilis muscle	2.4 g resveratrol per kg diet for 16 weeks.	↓ gains in BW ↑ *NQO1*, *GCLC*, and *HMOX1*mRNA	Ungvari et al., 2010
in vitro studies				
Cell culture	HCAEC treated with high glucose	10^{-6} – 10^{-4} mol/l for 24 h	↓ high glucose-induced mtROS production	Ungvari et al., 2009
	HCAEC treated with high glucose	10^{-7} – 10^{-4} mol/L for 24 h	— vascular ROS production to control levels ↓ high glucose-induced mitochondrial O2•- production	Ungvari et al., 2010
	BAEC	0.01, 0.1, 1.0, 10 μM for 24 h	↓ p47phox ↓ high glucose-induced ROS production	Tang et al., 2012
	HUVEC treated with H_2O_2	50 μM for 48 h	↓ H_20_2-induced down-regulation of SIRT1 ↓ SA-β-gal-positive cells ↓ ROS production /senescence process	Kao et al., 2010
	HUVEC	1 μM for 6 days	↑ eNOS and SIRT1 mRNA	Takizawa et al., 2013

			↑ G*ABARAP*, *LC3II* and *AGT3, MT1X* and *ANXA2* genes	
	HUVEC treated with TNF-α	10 μM for 2 h	↓ SQSMT1, ICAM1, PTGS2 and MMP9 protein levels ↑ SIRT1, LC3B2 and cAMP concentration ↑ MAP1LC3B2–to-actin ratio	Chen et al., 2013

Increased (↑) Reduced (↓); Maintained (—): Inhibited (/); High-fat/sucrose diet (HFS); Human coronary arterial endothelial cells (HCAEC); Human umbilical vein endothelial cells (HUVEC); Bovine aortic endothelial cells (BAEC).