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The Effect of Wild Blueberries on Endothelium-Dependent Vasodilation in Spontaneously Hypertensive Rats

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THE EFFECT OF WILD BLUEBERRIES ON ENDOTHELIUM-DEPENDENT VASODILATION IN SPONTANEOUSLY HYPERTENSIVE RATS

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An Abstract of the Thesis Presented
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The effect of wild blueberries on major endothelium-dependent vasodilation pathways and arterial blood pressure (BP) was examined in the young adult Spontaneously Hypertensive rat (SHR), used as a model of endothelial dysfunction, and the Wistar Kyoto (WK) rat, with functional endothelium, used as the control. Male SHR and WK rats were fed a control (SHR-C and WK-C), or a wild blueberry-enriched (SHR-B and WK-B) diet for nine weeks. By the age of 21 weeks, thoracic aortae were excised and 3mm arterial rings were prepared and immersed in Radnoti tissue baths. Rings were precontractred with phenylephrine (Phe) (10^{-6}M), followed by cumulative acetylcholine (Ach) doses (10^{-9}M to $3x10^{-6}$M) to generate dose-response curves in the absence or in the presence of either a nitric oxide synthase (NOS) inhibitor (L-NMMA at 10^{-4}M), a cyclooxygenase (COX) inhibitor (MFA at 10^{-5}M) or both inhibitors added simultaneously. The maximum Ach-induced vasodilation force (Fmax) and vessel sensitivity (pD_2) were determined for each treatment group.

A two-way analysis of variance (ANOVA) demonstrated no significant difference in the Fmax between the WK-B and WK-C groups. However, wild blueberries were found to reduce the pD_2 in response to Ach in the young adult WK rat (WK-B: 7.41 ± 0.02 vs. WK-C 7.49 ± 0.02, $p \leq 0.05$, n=9). In the young adult SHR, wild blueberries were shown to reduce Fmax in response to Ach (SHR-B: 92.13 ± 0.56 vs. SHR-C: 94.63 ± 0.56, $p \leq 0.05$, n=10). This effect is mediated by the COX pathway, as shown by the increased Fmax in response to Ach with the COX-pathway inhibition (SHR-B: 102.17 ± 0.57 vs. SHR-C: 97.76 ± 0.57, $p \leq 0.05$, n=10). Furthermore, wild blueberries were shown to increase the pD_2 of the young adult SHR aorta, when COX and NO pathways were inhibited separately, SHR-B: 7.72 ± 0.02 vs. SHR-C: 7.63 ± 0.02, $p \leq 0.05$, n=10, and SHR-B: 7.17 ± 0.02 vs. SHR-C: 7.04 ± 0.02, $p \leq 0.05$, n=10, respectively. Finally, wild blueberries did not have a significant effect on the systolic, diastolic or mean arterial BP in either strain.

Hence, the effect of wild blueberries on vasodilation depends on the physiological state of the aorta (functional vs. dysfunctional endothelium), as shown by the differential effect upon the functional endothelium of WK rat and dysfunctional endothelium of SHR. It is possible that the beneficial effect of wild blueberries on Ach-induced vasodilation may be masked by the high activity of COX-derived vasoconstrictor factors in the SHR model of endothelial dysfunction. Additionally, the effect of wild blueberries on vasodilation is not associated with blood pressure in the young adult SHR and WK rat.

Further studies are required to determine whether these observations are model-specific or reflect a general effect of wild blueberries on vasodilation during endothelial dysfunction.

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CHAPTER 1

INTRODUCTION

Cardiovascular disease (CVD) claims more lives each year than cancer, chronic lower respiratory diseases, accidents, and diabetes mellitus combined, with one in three American adults having one or more types of CVD. Furthermore, the economic impact of CVD is not to be ignored; only for 2007, the estimated direct and indirect cost of CVD was $431.8 billion (Rosamond *et al.*, 2007). In patients with all types of CVD, including coronary heart disease (CHD), peripheral arterial disease, chronic heart failure, and stroke, the vascular endothelium is the primary site of dysfunction (Brown *et al.*, 2001).

Since abnormal endothelial function is an early marker of CVD, the endothelium appears to be an ideal target for preventive therapy. Emerging evidence suggests the important role dietary factors play in modulating endothelial function in patients at risk and those with existing CVD. Various dietary components are hypothesized to influence CVD risk; antioxidants and flavonoids in particular, are favorably associated with vascular health (Brown *et al.*, 2001).

Blueberries contain anthocyanins, polyphenols and flavonoids and have the highest antioxidant capacity among tested fruits and vegetables (Prior *et al.*, 1998). Previous studies conducted in our laboratory demonstrated the positive effect of wild blueberries on endothelial function. In a study using young male normotensive Sprague-Dawley (SD) rats, wild blueberries incorporated into the diet, affected the vascular smooth muscle contractile machinery by suppressing the α-1 adrenergic receptor-agonist-mediated contraction through an

endothelium-dependent pathway (Norton *et al.*, 2005). In another study, wild blueberry consumption altered the structure of the extracellular matrix (EC) of male SD rat aortas, by increasing the concentration of glycosaminoglycans (GAGs) and decreasing the sulfation of all GAG type molecules, suggesting a possible effect of wild blueberries on endothelial and vascular smooth muscle signal transduction pathways (Kalea *et al.*, 2005). In addition, results of a more recent study suggested that wild blueberries affected the endothelium-dependent vasodilation of rat aorta by modulating cell membrane-agonist interactions in response to acetylcholine (Ach) in both young male SD rats and spontaneously hypertensive rats (SHR). Wild blueberries affected the endothelial-dependent vasodilation in SHR aorta most likely, by modulating a key pathway of endothelial function, the cyclooxygenase (COX) pathway (Clark, 2007).

In the proposed research, the effect of wild blueberries on endothelium-dependent vasodilation will be further investigated in **young adult** male SHRs and their normotensive control, the Wistar Kyoto (WK) rats.

The ***goal*** of this project is to study the effect of wild blueberries on the aortic functional properties of **young adult** hypertensive (SHRs) and normotensive (WK) rats, and to further investigate the mechanism(s) by which wild blueberries exert their action on the endothelium-mediated vasodilation of rat aorta.

The ***objectives*** are to determine:

1. whether wild blueberries affect Ach-induced endothelium-mediated vasodilation in young adult, 21 weeks of age, SHR and WK rats, and

2. the mechanisms of wild blueberry action on the COX and nitric oxide (NO) pathways by the use of COX and NO synthase (NOS) inhibitors, mefenamic acid (MFA) and L-NG-mono-methyl (L-NMMA) respectively, and

3. to determine the effect of wild blueberries on blood pressure (BP) regulation

This study is unique, because it is the first *ex vivo* dietary study to date that attempts to elucidate the effect of wild blueberries on endothelial function in **young adult** hypertensive and normotensive animals and probe their mechanisms of action on the COX and NO pathways. The SHRs will be utilized as a model of endothelial dysfunction that by the age of 21 weeks has developed full-blown hypertension. The WK rat will be used as a model of functional endothelium (control). Results from this project will further clarify the relationship between endothelial dysfunction and CVD and determine the potential use of wild blueberries on CVD prevention and/or therapy.

CHAPTER 2

LITERATURE REVIEW

2.1. Cardiovascular Disease and Endothelial Dysfunction

2.1.1. Endothelial Function

After the discovery of Furchgott and Zawadzki that Ach requires the presence of endothelial cells to elicit vasodilation (Furchgott and Zawadzki, 1980), the endothelial cell layer has gained all the more greater appreciation. The endothelium is now considered an indispensable organ that regulates the vascular tone and maintains the vascular homeostasis in a paracrine, endocrine and autocrine fashion. A healthy endothelium promotes vasodilation and also has antioxidant, anti-inflammatory, anticoagulant and profibrinolytic properties. Additionally, leukocyte adhesion and migration, as well as vascular smooth muscle cell (VSMC) proliferation and migration are inhibited by the endothelium under normal physiological conditions (Bonetti *et al.*, 2003).

The endothelium, located in the interface off the vessel wall and the blood stream, releases substances that regulate vasomotor function, trigger inflammatory processes and affect vascular homeostasis, in response to pressure, shear force or vasoactive factors (Endemann and Schiffrin, 2004).

The endothelium contributes to the physiological regulation of vasomotor control through maintaining equilibrium in releasing endothelium-derived relaxing factors (EDRF) and endothelium-derived contracting factors (EDCF). The EDRF such as nitric oxide (NO), prostacyclin or prostaglandin I_2 (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF) counteract the effect of the EDCF, such as

endothelin-1 (ET-1), thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2) in maintaining a physiological vascular tone (Vanhoutte, 2003). Two major biochemical pathways of the endothelium that determine the vascular tone are the NO synthesis pathway and the COX pathway, which generates both vasodilator and vasoconstrictor prostanoids via the metabolic conversion of arachidonic acid (AA) (Moncada and Higgs, 2006).

2.1.1.1. Vasodilators

The principal vasodilatory substance released by the endothelial cells is NO, a free radical molecule, which was discovered to be a potent vasodilator (Vallance *et al.*, 1989). At the vascular level, NO, which is chemically equivalent to EDRF (discovered by Furghott and Zawadski, 1980), mediates endothelium-dependent relaxation of vascular smooth muscle (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). Nitric oxide is a key signaling molecule in physiological and pathological processes; both NO and its reactive nitrogen species products are implicated in all aspects of normal and disease conditions (Mollace *et al.*, 2005). Nitric oxide is formed in the endothelial cells from the guanine-nitrogen terminal of L-arginine, by the constitutive endothelial NOS (eNOS, NOS III) via an oxidative reaction that yields NO and citrulline. Endothelial NOS is Ca^{2+}-calmodulin-dependent, i.e. its activation depends on the intracellular concentration of calcium ions in the endothelial cell. The activity of the enzyme requires cofactors such as nicotinamide-adenine-dinucleotide phosphate (NADPH) and 5,6,7,8-tetra-hydrobiopterin (BH_4). Endothelial NO synthase can be inhibited competitively by

L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro - L-arginine (Vanhoutte, 2003). The identification of L-NMMA as an inhibitor of the synthesis of NO provided the most important pharmacological tool for investigating the presence and roles of NO in biological systems (Rees *et al.*, 1989).

Nitric oxide generation via the NOS pathway does not occur solely in the vascular wall, indicating the biological significance of NO molecule in other systems as well. So far, NO has been primarily studied with regard to cardiovascular, nervous, and the immune system. There are three major isoforms of NOS, originally named after the tissues in which they were first identified: two calcium/calmodulin dependent; constitutive isoforms, eNOS and neuronal NOS (nNOS, NOS I), and calcium-independent, inducible NOS (iNOS, NOS II), which is expressed in macrophages and other tissues following immunological stimulation (Moncada and Higgs, 2006).

It is now well established that eNOS is important for cardiovascular homeostasis, vessel remodeling, and angiogenesis. Endothelial NOS cellular location favors high local concentrations of NO in the vicinity of circulating blood cells and vascular smooth muscle (Fulton *et al.*, 2001).

Nitric oxide is a major vasodilator in large arteries such as the coronary, systemic, mesenteric, pulmonary and cerebral arteries, indicated by the finding that NOS inhibition results in vasoconstriction and an increase in systemic arterial pressure in both animals and humans (Vanhoutte, 2003).

Besides the fundamental role in vasodilation, NO inhibits VSMC growth, platelet and leukocyte adhesion to the endothelium, and prevents the production of the vasoconstrictor ET-1 (Vanhoutte, 2003).

Physical and humoral stimuli regulate NO production. The shear stress exerted by blood flow on the endothelial cell is one of the main factors that determines the local release of NO and thereby the flow-dependent vasodilation (Vanhoutte, 2003). The endogenous substances that stimulate the release of NO include catecholamines, vasopressin, bradykinin, histamine, serotonin or adenosine diphosphate (ADP) released by aggregating platelets, or thrombin, formed during blood coagulation (Vanhoutte, 2003). These hormones and autocoids, activate endothelial cell membrane receptors, coupled to different G-proteins, to induce NO generation (Vanhoutte, 2003). Once synthesized, NO diffuses to the VSMC and induces its relaxation via a cascade of events that starts with activation the cytosolic enzyme soluble guanylate cyclase (sGC). Soluble GC catalyzes the production of cyclic 3, 5-guanosine monophosphate (cGMP). Cytosolic Ca^{2+} removal from the cell and inhibition of the contractile apparatus follows the activation of a cGMP-dependent protein kinase G. The action of protein kinase G has direct influence on the phosphorylation of gap junctions and activity of potassium and calcium channels. Phosphorylation of potassium channels causes K^+ outflow from the cell, while phosphorylated calcium channels decrease Ca^{2+} influx. Calcium release from calmodulin, due to the reduced cytoplasmic Ca^{2+}-concentration, leads to dephosphorylation of myosin light chain, which prevents

myosin head binding to actin and thereby produces relaxation of smooth muscle (Lincoln *et al.*, 1994).

In contrast, iNOS is calcium independent and once expressed produces NO in large amounts for a prolonged period of time (Nathan, 1992). Inducible NOS is thought to mediate the vast majority of pathological effects attributed to NO, having a fundamental role in the inflammatory process (Mollace *et al.*, 2005).

Another important vasodilator secreted by the endothelium is PGI_2, a major product of arachidonic acid (AA) metabolism (Moncada *et al.*, 1979). Cyclooxygenase or prostaglandin endoperoxide synthase (PGHS) is the key enzyme of AA conversion into prostanoids that starts with AA release from the cell membrane phospholipids under the action of phospholipase A_2 (PLA_2), followed by its conversion, first into the cyclic endoperoxide prostaglandin G_2 (PGG_2) and subsequently into the endoperoxide prostaglandin H_2 (PGH_2), with both steps catalyzed by COX. These unstable intermediate products of AA metabolism are rapidly converted into the biologically active prostaglandins PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TxA_2 via specific synthases (Mollace *et al.*, 2005). Two distinct COX enzymes have been identified to date, COX-1, the constitutive form found in virtually all organs, and COX-2, the inducible form detected under inflammatory conditions in various cells (Mollace *et al.*, 2005). The ubiquitous COX-1 has clear physiological functions. Basal COX-1 activation is responsible for PGI_2 production and therefore the vasodilatory, antithrombotic and cytoprotective effect of this prostanoid (Moncada *et al.*, 1976). In contrast, COX-2 is induced by proinflammatory cytokines and growth factors and expressed in

many cells during inflammatory processes (e.g. macrophages, monocytes, fibroblasts (Mollace *et al.*, 2005).

Prostacyclin was first described as an EDRF in 1979 (Moncada *et al.*, 1979). The vasodilation and inhibition of platelet aggregation induced by PGI_2 are correlated with an activation of the adenylyl cyclase (AC), leading to a rise in intracellular cyclic adenosine monophosphate (cAMP) (Moncada *et al.*, 1987). The vasorelaxing effect of prostacyclin is determined by the specific VSMC relaxant prostacyclin receptors (IP), which mediate an increase in cAMP. Cyclic AMP induces vascular smooth muscle relaxation by reducing intracellular Ca^{2+} levels and inhibiting of myosin light chain kinase (MLCK), the enzyme that phosphorylates myosin and induces contraction (Narumyia *et al.*, 1999). The effect of NO and PGI_2 in VSMC relaxation appears to be connected. The increase of both cGMP and cAMP induces VSMC relaxation by reducing intracellular Ca^{2+} concentrations (Lincoln *et al.*, 1990). Cyclic GMP has been proposed to enhance the accumulation of cAMP, due to the cGMP-mediated inhibition of phosphodiesterase enzyme which degrades cAMP (Maurice *et al.*, 1991). The above mechanism also mediates the NO and PGI_2 interaction for the inhibition of platelet aggregation, with the cGMP promoting cAMP accumulation in the platelets (Maurice and Haslam, 1990).

Prostaglandin E_2 (PGE_2) is another vasodilatory factor which produces smooth muscle relaxation through interaction with the EP_2 and EP_4 relaxant prostanoid receptors that are responsible for cAMP increase (Narumiya *et al.*, 1999).

Yet, another factor that contributes to the endothelium-dependent relaxation causing hyperpolarization in response to Ach and other vasodilators is the endothelium-derived hyperpolarizing factor (EDHF), a diffusible substance, different from NO and PGI_2. The exact nature of EDHF still remains to be determined, while several candidates such as epoxyeicosatrienoic acids (EETs), potassium ions and hydrogen peroxide have been proposed (Feletou and Vanhoutte, 2000).

Accumulating evidence indicates that a constant cross-talk occurs in the endothelium between NO and prostanoids. Produced at low levels, NO appears to regulate the COX pathway towards COX-derived vasodilator release (Salvemini *et al.*, 1996). There is also an indirect relationship between NO and prostanoids in the VSMC, due to the interaction of cAMP with cGMP in the phosphodiesterase pathway (Delpy *et al.*, 1996). Under normal physiological conditions, the basal release of NO and PGs by constitutive NOS and COX respectively, has been shown to protect against vascular diseases via enhanced vasodilation and antiplatelet activity (Mollace *et al.*, 2005). The increase in endogenous NO following treatment with L-arginine may enhance the vasodilator PG level. Moreover, the enhanced release of NO may compensate for the attenuation of prostacyclin production that follows COX-inhibition. Contrastingly, the vasodilator PGs seem to have no capacity to modulate NO (Vassale *et al.*, 2003). However, in the presence of reduced NO availability, alternative pathways, including hyperpolarization, account for endothelium-dependent vasodilation (Taddei *et al.*, 1999).

2.1.1.2. Vasoconstrictors

The role of the endothelium in vascular tone regulation, besides relaxing factors, entails the generation of vasoconstricting factors (Furghott and Vanhoutte, 1989). These vasoconstricting factors include AA metabolites such as TXA_2 and PGH_2, reactive oxygen species (ROS), ET-1 and angiotensin II (Heymes *et al.*, 2000).

The first observations of endothelium-dependent contractions were made in canine arteries, where AA and thrombin, which are endothelium-dependent dilators in isolated arteries, potentiated endothelium-dependent contraction mediated by α1-adrenoreceptor agonist. This endothelium-mediated response was attributed to the release of a diffusible factor(s) named EDCF (De Mey and Vanhoutte, 1982). Incubation of canine veins with COX inhibitors could prevent the endothelium-dependent contractions in response to catecholamines (Miller and Vanhoutte, 1985). Inhibitors of COX were also shown to prevent endothelium-dependent contractions or to normalize endothelium-dependent relaxations in various arterial preparations (Katusic *et al.*, 1988; Luscher and Vanhoutte, 1986; Miyamoto *et al.*, 1999), leading to the conclusion that COX product(s) play a key role in the EDCF-mediated responses (Vanhoutte *et al.*, 2005).

Cyclooxygenase mediated contractions so far are attributed to oxygen free radicals, mainly superoxide anions (O_2^-), generated by the hydroperoxidase activity of the enzyme, and prostanoids such as TXA_2 or PGH_2 (Vanhoutte *et al.*, 2005). These vasoconstrictor prostanoids act through activating the endoperoxides/ thromboxane receptors (Narumyia *et al.*, 1999). While

prostanoids act exclusively as direct vasoconstrictors, oxygen free radicals have a direct vasoconstricting effect, possibly via PGH_2 receptors, but also act indirectly by compromising NO bioavailability (Taddei *et al.*, 2003).

Endothelin is a potent vasoconstrictor released by the endothelium to oppose the vasodilatory effect of NO. Endothelin contribution to vascular tone is mediated by the endothelin receptors ETA and ETB, which trigger the phosphatidylinositol pathway and thereby Ca^{2+} release from intracellular stores and vasoconstriction (Marasciulo *et al.*, 2006). Under physiological conditions ET-1 is highly regulated via inhibition or stimulation from endothelium. Factors such as shear stress or thrombin, epinephrine, angiotensin II and free radicals enhance ET-1 release, whereas mediators such as NO, PGI_2 and cGMP attenuate ET-1 generation. However, with endothelial dysfunction and the subsequent decrease in NO bioavailability, ET-1 synthesis, release or activity is relatively augmented (Marasciulo *et al.*, 2006).

Overall, vascular homeostasis depends on the balance of the bioactive factors released by the endothelium. A dysfunction of the endothelial cell disrupts this balance and leads to so called endothelial dysfunction (Verma and Anderson, 2002).

2.1.2. Endothelial Dysfunction

Endothelial dysfunction is a condition characterized by a shift in the actions of the endothelium toward reduced vasodilation, proinflammatory conditions and prothrombotic properties. It is associated with most forms of CVD, such as

hypertension, coronary heart disease, chronic heart failure, peripheral heart disease, diabetes and chronic renal failure (Endeman and Schiffrin, 2004). Endothelial dysfunction, presenting a systemic nature, plays a major role in the development and progress of the atherosclerotic processes (Bonetti *et al.*, 2003). Endothelial dysfunction constitutes an independent predictor of cardiovascular events (Vita and Keaney, 2002), by predisposing the vessel wall to vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, oxidative stress, thrombosis, impaired coagulation, inflammation and development of atherosclerotic lesions (Verma and Anderson, 2002).

The dysfunction of the endothelial cells is evidenced by impairment in endothelium-dependent relaxation, mainly due to a reduced release of EDRF, and NO in particular, although EDCF production may also contribute (Vanhoutte, 2003). Nitric oxide reduction and the subsequent endothelial dysfunction seem to precede any other evidence of CVD in humans with a family history of atherosclerosis risk factors such as essential hypertension (Moncada and Higgs, 2006). Reduced activity of eNOS due to endogenous or exogenous inhibition, reduced L-arginine availability and reactive species that attenuate NO bioavailability, adversely affect NO levels (Endeman and Schiffrin, 2004). Nitric oxide has been shown to regulate the synthesis of prostanoid vasodilators such as PGI_2, as well as vasoconstrictors such as TXA_2 and PGH_2, affecting the ratio between vasodilator and vasoconstrictor prostanoids. Changes of vasodilator to vasoconstrictor ratio are important for the development of vascular dysfunction (Shimokawa, 1999).

Endothelial dysfunction accompanied by the increased production of vasoconstrictor seems to play a key role in the progression of CVD (Vanhoutte *et al.*, 2005). Both animal and human data so far, suggest that the production of COX-dependent EDCF is a major mechanism that leads to an impaired availability of NO, at least in age-related or essential hypertension (Vanhoutte *et al.*, 2005). Additionally, increased vascular ROS play an important role in the process leading to endothelial dysfunction (Cai and Harrison, 2000). For instance, ROS generated by COX, reduce the biological activity of NO directly and indirectly by contributing to lipid peroxidation, products of which might also decrease NO synthesis and bioavailability (Keaney and Vita, 1995; Sherman *et al.*, 2000). Major predisposing conditions to atherosclerosis present an increased vascular superoxide production (Cai and Harrison, 2000). Enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase have been implicated in the superoxide generation in the vasculature, especially under pathological conditions that trigger a greater activity and expression of these enzymes (Cai and Harrision, 2000). Another source of O_2^- in the vasculature is the vascular cytochrome P_{450} enzymes. Inhibition of these enzymes appears to improve endothelium-dependent vasodilation mediated by NO in patients with coronary heart disease (Cai and Harrision, 2000).

The activity of NO is deteriorated through the formation of peroxynitrite anion ($ONOO^-$), generated by the reaction of O_2^- with NO (Landmesser *et al.*, 2004). Peroxynitrite anion is a powerful oxidant species implicated in established clinical conditions such as hypercholesterolemia, diabetes and coronary heart disease

(Greenacre and Ischiropoulos, 2001). In such disorders an increased ROS formation is observed in blood vessels. Treatment with antioxidants enhances endothelium dependent-vasodilation in the forearm and the coronary circulation of individuals with coronary artery disease and diabetes (Moncada and Higgs, 2006). Moreover, peroxynitrite leads to degradation of the eNOS cofactor, BH_4 and therefore eNOS "uncoupling". The "uncoupling" of eNOS is the process of activation of the reductase function of the enzyme leading to ROS formation (Landmesser *et al.*, 2003), reported to occur in several pathological conditions such as diabetes, hypercholesterolemia and hypertension (Cai and Harrison, 2000; Moncada, 2006). Additionally, $ONOO^-$ plays an important role in regulating the COX pathway enzymes. It can increase both COX-1 and COX-2 activity, modify AA yielding PGF_2-like compounds with powerful vasoconstrictor effects and also attenuate PGI_2 production (Mollace *et al.*, 2005).

In general, a reduced level of vasodilators, mainly NO, combined with an increased vasoconstrictor activity, attributed to both COX-derived vasoconstrictor prostanoids and ROS, disrupts the homeostasis of endothelial cell and lead to an impairment of the endothelium dependent relaxations, which is a basic feature of endothelial dysfunction.

2.1.2.1. Spontaneously Hypertensive Rat as a Model of Endothelial Dysfunction

The SHR has been broadly used as a model of essential hypertension and endothelial dysfunction not only in pharmacological studies (Luscher and

Vanhoutte, 1986; Xiao and Pang, 1994; Yang *et al.*, 2002), but in dietary studies as well (Duarte *et al.*, 2001; Machha *et al.*, 2005; Rodriguez-Iturbe *et al.*, 2003). The strain of SHRs was developed in 1963 from outbred WK rats (Okamoto and Aoki, 1963). The SHR develops hypertension spontaneously with no exception at the age of 7 to 15 weeks. The systolic blood pressure (BP) plateaus at approximately 200 mmHg (Yamori, 1984). The age of 10 weeks in SHR is considered as the early hypertensive stage, with a systolic BP at approximately 170 mmHg (Tanase *et al.*, 1982). The increased peripheral vascular resistance of the SHR is mostly determined by neurogenic mechanisms related to a disorder of central BP regulation. Structural changes in the vasculature of SHR, due to the increased BP and neurogenic tone, contribute to the maintenance of the hypertension. In the SHR, VSMCs seem to be genetically predisposed to hyperplastic growth and β-adrenergic stimulation (Yamori, 1984). Additionally, SHRs present increased levels of noraderanline, but not of total catecholamines (Grobecker *et al.*, 1975), as well as an abnormal electrolyte balance (Dietz *et al.*, 1984). In the SHR, environmental and dietary factors can influence the degree of hypertension (Yamori, 1984). Caloric restriction has been observed to lower BP (Young et al., 1978), while a high salt diet seems to increase systolic BP (Adams and Blizard, 1991).

The endothelial dysfunction and the high BP in the SHR is also attributed to decreased NO availability, enhanced oxidative stress and an overall increased formation of COX-derived EDCF that lead to inhibition of endothelium-dependent vasodilation (Vanhoutte, 2003). The pharmacological concentrations of Ach

($3x10^{-7}$ to $3xI0^{-5}$M) can cause endothelium dependent contraction in the aorta of the SHR, but not in the normotensive rats, suggesting an impaired production and/or diminished bioavailability of NO in essential hypertension (Luscher and Vanhoutte, 1986). Endothelial NOS activity seems to be preserved, and even increased in the SHR, probably as a compensatory mechanism for the increased BP (Xiao and Pang, 1994). However, the NO bioavailability is attenuated; further so due to an increased superoxide generation by NADPH. Therefore, the endothelium-dependent vasodilation is impaired in the SHR (Zalba *et al.*, 2001, a). The reduced NO bioavailability amplifies the endothelium-dependent contractions (Yang *et al.*, 2002; 2003).

Product(s) of COX pathway play a key role in vasoconstriction, since the COX inhibitor indomethacin seems to normalize endothelium-dependent relaxations in the aorta of the SHR (Luscher and Vanhoutte, 1986; Ito *et al.*, 1991). Preferential inhibitors of COX-1, but not of COX-2 prevented the endothelium-dependent contractions to Ach (Ge *et al.*, 1995; Yang *et al.*, 2002). The constitutive isoform of COX is responsible for the blunted endothelium-dependent relaxation to Ach in the SHR aorta, suggested by the finding that COX-1 expression was significantly greater in the aorta of adult SHRs than in that of normotensive WK controls (Ge *et al.*, 1995). Although indomethacin cancelled the Ach-induced endothelium-dependent contraction in the SHR, TXA_2 synthase selective inhibition, did not modulate endothelium dependent contraction, suggesting that some AA product(s), other than TXA_2, are responsible for the vascular contractions in the SHR (Luscher and Vanhoutte, 1986). The COX-derived EDCF could be the

unstable endoperoxides that produce contraction by stimulating the TXA_2/PGH_2 receptors on the VSMC, since the inhibition of TP receptors was shown to improve to some extent the vasodilation in the SHR (Luscher and Vanhoutte, 1986). Prostacyclin has been also implicated, at least in part, in the endothelium-dependent contractions induced by Ach in the SHR aorta. This particular effect of PGI_2 in the SHR seems to be mediated through the activation of TP receptors in the VSMC (Gluais *et al.*, 2005). Prostacyclin, acting as a vasoconstrictor, was also found to be the main culprit for the endothelial dysfunction in the SHR aorta treated with aldosterone. In the same study, other prostanoids were also involved in the aldosterone-induced endothelial dysfunction in the WK rat (Blanco-Rivero *et al.*, 2005).

Besides prostanoids, COX-derived ROS seem to contribute to the pathogenesis of endothelial dysfunction in experimental hypertension (Katusic and Vanhoutte, 1989; Yang *et al.*, 2002). The increased O_2^-, may mediate Ach-induced endothelium-dependent contractions and endothelial dysfunction in the SHR (Kerr *et al.*, 1999; Yang *et al.*, 2002; Cuzocrea *et al.*, 2004). Superoxide anions can stimulate COX-1 to convert AA into endoperoxides, which activate TP-receptors of the vascular smooth muscle. A greater activity of COX-1 and a greater TP-receptor response are both required for endothelium-dependent contractions (Ge *et al.*, 1995; 1999).

The endothelium-dependent contractions can be depressed by scavenging or depleting superoxide anions (Yang *et al.*, 2002; 2003). Furthermore, anti-oxidant

dietary treatment has been shown to improve endothelial function in the SHR (Maccha and Mustafa, 2005; Rodriguez-Iturbe *et al.*, 2003; Ulker *et al.*, 2003).

An important factor that determines the impaired vasodilation and the progression of endothelial dysfunction in the SHR is age. In SHR younger than 14 weeks, the endothelium-mediated vasorelaxation seems to be similar to normotensive rats of the same age. Hence young SHRs can be considered as normotensive before full development of hypertension (Cappelli-Bigazzi *et al.*, 1997). The aging effect on the old SHR and WK rat aorta is reflected through various changes in the endothelium and smooth muscle as well. While in the young adult SHR (12 to 14 weeks), PGH_2 accounts for the reduced endothelium-dependent relaxation, in old (72 weeks) WK rat and SHR, reduced NO levels, due to either impaired formation or increased inactivation, seem to be involved (Kung and Luscher, 1995). Age, does not seem to affect contractions of ET-1 in the WK rat, whereas the response is diminished in 72 week vs. 12 week-old SHR (Kung and Luscher 1995). In 30-week adult SHRs, the reduced NO availability induced by NADPH superoxide production seems to play a critical role in the impaired endothelial function in comparison with the 14-week old SHR (Zalba *et al.*, 2001, b). Furthermore, in one year old SHR, PGI_2 released by Ach was shown to act as a contracting and not a relaxing factor (Gluais *et al.*, 2005), probably due to a decreased response of the IP receptor. In the aorta of older than 15-week WK or SHR, IP receptor agonists cannot evoke relaxations (Levy, 1980; Rapoport and Williams, 1996). The expression of the IP receptor decreases with age, but in the SHR the receptor is less expressed than in the

WK rat at any age (Numaguchi *et al.*, 1999). The endothelium-dependent contraction in response to Phe is increased, whereas the endothelium-dependent relaxation is reduced in aging SHR (12 weeks vs. 16 months). Alterations in the sGC-NO pathway are involved in the age-related changes in vascular contractions and relaxations. However, long-term treatment with vitamins E and C or tempol, a superoxide dismutase (SOD) mimetic, was shown to partially reverse the age-related inhibition of vascular relaxation (Payne *et al.*, 2003). Acetylcholine-mediated vasorelaxation, even after treatment with a non-selective COX inhibitor, did not differ significantly among WK rats and SHR between 3 to 6 months of age, while the release of COX-dependent vasoconstrictors occurred only in vessels of aged (12 to 25 months) normotensive or SHR animals and in response to higher concentrations of Ach (Koga *et al.*, 1989). Treatment with simvastatin, an inhibitor of the rate-limiting enzyme in the cholesterol biosynthesis, was associated with reduced COX-1, but not COX-2 expression in the aorta from aged WK rat and SHR. The capacity of the aged aorta to release COX-2-derived vasoconstrictors was reduced, whereas PGI_2 release was not altered with the simvastatin treatment (de Sotomayor *et al.*, 2005). In support of the above finding, COX-1 expression was significantly increased in aortas of the older WK rats and SHRs (Ge *et al.*, 1995; 1999).

Therefore, the aging process in the SHR is characterized by a further deterioration of endothelial dysfunction, adversely affecting NO bioavailability and shifting the COX pathway effect on vasomotion towards an increased vasoconstrictor and a reduced vasodilator activity.

Despite the complexity of the endothelial defects of the SHR, this animal model is considered suitable to study endothelial dysfunction and essential hypertension, due to the great similarity the SHR shows with humans with essential hypertension.

2.1.2.2. Endothelial Dysfunction in Essential Hypertension

Essential or primary hypertension is a multifactorial and complex disease not well-understood so far. However, a strong association of essential hypertension with endothelial dysfunction has been documented (John and Schmieder, 2000). Similar to experimental hypertension, endothelium NO-dependent vasodilation is impaired in human hypertension as well. Administration of L-arginine can increase the vasodilating effect of Ach in normotensive patients, but not in essential hypertensive patients (Taddei *et al.*, 1997). In addition L-NMMA can blunt the response to Ach in normotensive, but not in hypertensive patients (Panza *et al.*, 1995). Endothelial dysfunction is attributable not only to a defect in the L-arginine-NO pathway (Taddei *et al.*, 1997), but to the production of COX-dependent EDCF(s) as well (Taddei *et al.*, 1998). In essential hypertensive patients, the vasodilation to Ach is blunted and not affected by inhibition of NO synthase (Dohi *et al.*, 1990). However, indomethacin was shown to increase and almost normalize the vasodilator response to Ach in these patients (Taddei *et al.*, 1998). These findings demonstrate that in essential human hypertension, vasoconstrictor products of COX are mainly responsible for the abnormal reaction to endothelium-dependent vasodilators (Vanhoutte *et al.*, 2005).

However, the production of COX-derived EDCF(s) may not be implicated in all forms of hypertension since COX inhibitors were shown to improve vasodilation in human subjects with essential hypertension, but not in secondary forms of hypertension (Taddei and Salvetti, 2002).

Oxidative stress seems to account for the impaired NO availability that characterizes the endothelial dysfunction associated with human essential hypertension. The antioxidant vitamin C was shown to improve the endothelium-dependent vasodilation by restoring NO availability in the forearm microcirculation of essential hypertensive patients (Taddei *et al.*, 1998).

The impaired endothelium-dependent vasodilation in essential hypertension may not necessarily be a causal mechanism responsible for the development or maintenance of increased BP. Available evidence seems to indicate dissociation between the degree of endothelial dysfunction and arterial BP values (John and Schmieder, 2000). Impaired endothelium-dependent vasodilation appears to be at least partially genetically pre-determined (Rossi *et al.*, 2003). Moreover it seems that there is no correlation between BP values and endothelium-dependent vasodilation (Panza *et al.*, 1993). Finally, BP reduction per se is not associated with improvement of endothelium-dependent vasodilation (Panza *et al.*, 1993; Taddei and Salvetti, 2002). Therefore, endothelial dysfunction, although associated with essential hypertension, may not be related to hemodynamic load.

On the other hand, endothelial dysfunction is not specific to essential hypertension, but commonly observed in connection with the major

cardiovascular risk factors (Vita *et al.*, 1990; Taddei and Salvetti, 2002). The exact relationship between endothelial dysfunction and cardiovascular events in essential hypertension patients remains to be elucidated (Taddei *et al.*, 2003).

Independent of essential hypertension, a continuous impairment of the endothelium occurs with age. In normotensive human subjects a defect in the NO pathway seems to be the main cause of age-related endothelium dysfunction as EDCF contribution is minimal up to 60 years of age. Above the age of 60, EDCF seems to participate in the process, while the defect on the NO pathway is aggravated (Taddei *et al.*, 1997). In contrast, the contribution of COX-derived vasoconstrictors to the endothelium impairment starts at an earlier age (31-45 years) and increases even more with age in humans with essential hypertension (Taddei *et al.*, 1997).

Vitamin C and indomethacin improved Ach-induced vasodilation in normotensive humans above the age of 60 years, indicating that COX-derived ROS contribute to the EDCF-mediated endothelium responses in aging and hypertension (Taddei *et al.*, 2001). The aging blood vessel wall is characterized by the production of COX-derived EDCF, but essential hypertension causes an earlier onset and a greater progress of the dysfunctional endothelium (Vahoutte *et al.*, 2005). Decreased NO synthesis was also shown in aged human umbilical vein endothelial cells (HUVECs), where upregulation of eNOS expression induced by shear stress was impaired. Furthermore, aging of the endothelial cells resulted in enhanced apoptosis due to the loss of eNOS expression (Hoffmann *et al.*, 2001).

Overall, a progressive reduced expression and activity of eNOS, as well as increased release of ROS and COX-derived contracting prostanoids alters the equilibrium between vasorelaxing and vasoconstricting endothelium-derived factors and results in the age-related endothelial dysfunction (Matz and Andriantsitohaina, 2003).

2.2. Wild Blueberry Composition and Bioactive Components

Wild blueberries (*Vaccinium angustifolium*) have exhibited one of the highest recorded *in vitro* antioxidant capacity among various fruits and vegetables tested (Prior *et al.*, 1998). Also, the wild blueberry has a higher *in vitro* antioxidant capacity than the cultivated highbush blueberry (*Vaccinium corymbosum*) (Kalt *et al.*, 2001). Consumption of blueberries has been associated with an increase of total serum anthocyanins and serum antioxidant status in human subjects (Mazza *et al.*, 2002; Kay and Holub, 2002). Prior *et al.* (1998) confirmed a direct relationship between the total oxygen radical absorbance capacity (ORAC) and the total phenolic content of several *Vaccinium* species *in vitro* (Prior *et al.*, 1998). The ORAC assay is a method developed to quantify the total antioxidant activity of a biological sample (Cao *et al.*, 1993).

Anthocyanins are among the most abundant flavonoids in wild blueberries (Kalt and McDonald, 1996). Five major anthocyanins were identified in the lowbush blueberry: malvidin, delphinidin, cyanidin, petunidin and peonidin (Table 2.1). Anthocyanins in wild blueberry are found as 3-glucosides, 3-galactosides and 3-arabinosides (Gao and Mazza, 1995). Chlorogenic acid is the major phenolic

acid in lowbush blueberries, while other major organic acids include citric, malic and quinic acids (Kalt and McDonald, 1996). The major flavonols detected in blueberries are quercetin and myricetin (Hakkinen *et al.*, 1999) (Table 2.1). Wild blueberries also contain proanthocyanins, which are higher molecular weight tannin components, shown to inhibit the initiation stage of chemically induced carcinogenesis (Smith *et al.*, 2000). The stilbene compound resveratrol is also found in lowbush blueberries (863 ng/g dry weight) (Rimando *et al.*, 2004). According to Bushway *et al.* (1983), wild blueberries contain a variety of vitamins and minerals. A 100g wet weight portion of wild blueberries offers approximately 7, 3, 2, 11 and 2% of the dietary reference intake (DRI) for niacin, riboflavin, thiamin, vitamin C and A respectively. With regards to mineral composition, the eleven elements of blueberries are calcium, potassium, magnesium, phosphorus, aluminium, boron, copper, iron, manganese, sodium and zinc. The DRI for these minerals are 3% or less, with the exception of manganese, which is found in wild blueberry composition at levels of 50-100% of the DRI, making them an excellent source of manganese (Bushway *et al.*, 1983).

In humans, the daily intake of the total antioxidants from fruit and vegetables was significantly correlated with the fasting plasma antioxidant capacity (ORAC) (Cao *et al.*, 1998). Since wild blueberries are relatively low in antioxidant vitamins and minerals (Bushway *et al.*, 1983), their *in vitro* antioxidant capacity has been attributed to their high concentration of phenolic compounds, particularly anthocyanins (Kalt *et al.*, 1999; Prior *et al.*, 1998; Smith *et al.*, 2000).

Table 2.1. Flavonoid Content of Blueberries

Anthocyanidins	Malvidin	49.21
	Delphinidin	29.54
	Cyanidin	15.02
	Petunidin	11.73
	Peonidin	7.05
Flavonols	Quercetin	3.11
	Myricetin	0.82

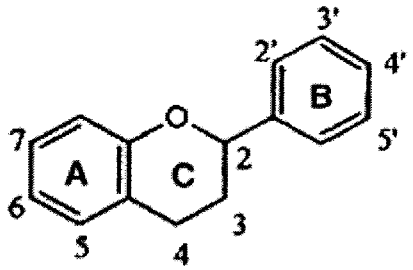
Adapted from USDA database for the flavonoid content of selected foods, 2003 (mg/100 g edible portion)

2.3. Flavonoids

2.3.1. Flavonoid Structure

Flavonoids comprise the most common and widely distributed group of the polyphenolic compounds that occur naturally in plants. The common structure of flavonoids consists of two aromatic benzene rings (A and B ring) linked through a three carbon oxygenated heterocycle (C ring) (Figure 2.1) (Bravo, 1998).

Figure 2.1. Basic Flavonoid Structure (flavan nucleus)



(Adapted from Erdman *et al.*, 2007)

Variations in the heterocyclic ring C account for the different classes of flavonoids, namely flavonols, flavones, catechins (flavan-3-ols), flavanones, anthocyanidins and isoflavonoids. Additionally, the basic structure of the flavonoid molecule allows for a wide range of substitutions in the benzene rings, A and B, within each class of flavonoids: phenolic hydroxyls, O-sugars, methoxy

groups, sulfates and glucorunides (Hollman and Katan, 1999). The hydroxyl groups of all rings are potential sites for linkage to carbohydrates. Flavonoids that have at least one sugar molecule comprise the flavonoid glycosides, whereas those that are not bound to a sugar molecule are called aglycones. Attachment of acetyl and malonyl groups to the sugar conjugates further increases the structural complexity of the flavonoids (Beecher, 2003). The most common form of flavonoids found in plants is the glycoside derivatives (Bravo, 1998). Glucose is the most typical glycosidic unit; other sugar units include glucorhamnose, galactose, arabinose and rhamnose (Cook and Samman, 1996).

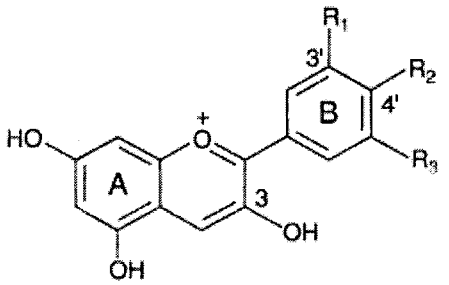
The chemical structure of flavonoids affects their metabolism and biological activity (Heim *et al.*, 2002). Sugar molecules or other functional groups attached to the basic flavonoid structure or flavan nucleus affect flavonoid absorption and metabolism (Hollman and Katan, 1999). The radical scavenging and chelating activities are influenced by the number, position and types of substitution molecules of the basic flavan nucleus (Heim *et al.*, 2002).

The antioxidant activity of flavonoids is mainly attributed to the presence of phenolic hydroxyl groups (Kandaswami and Middleton, 1994). The B-ring hydroxyl group in particular is the most significant factor for ROS scavenging ability. Additionally, the resonance of electrons between the A and B rings is an important determinant of antioxidant and other biological activities of flavonoids (Burda and Oleszek, 2001). Recent findings suggest that the structural features required for antioxidant activity may be unrelated to those needed for anti-inflammatory activity of compounds such as quercetin and related flavonoids

(Loke *et al.*, 2008). The 2,3 double bond of the C-ring is essential for leukotriene inhibitory activity (Loke *et al.*, 2008), as well as inhibition of adhesion molecule expression in endothelial cells (Lotito and Frei, 2006) whereas the absence of the C-3 hydroxyl group significantly reduces antioxidant properties (Loke *et al.*, 2008).

Anthocyanins are distinguished from other flavonoids as a separate class due to their ability to form flavylium cations (Prior *et al.*, 2006) (Figure 2.2).

Figure 2.2. Basic Anthocyanin Structure (flavylium cation)



(Adapted from Prior *et al.*, 2006)

Anthocyanins (Greek: anthos = flower and kyanos = blue), are the water soluble colorful compounds that provide the red, purple and blue colors of many vegetables and fruits. The structural variations of anthocyanins stem from differences in the number of hydroxyl groups, the degree of methylation of these hydroxyl groups, the nature and the number of the sugar moiety attached to the phenolic (aglycone) molecule and the position of attachment, and finally the nature and the number of aliphatic or aromatic acids attached to the sugars (Kong *et al.*, 2003) (Table 2.2). The basic anthocyanin structure is comprised of the aglycone part, named anthocyanidin, and a sugar moiety mainly attached at the 3-position on the C-ring or the 5, 7-position on the A-ring (Prior *et al.*, 2006).

Table 2.2. Chemical Structure of the Major Blueberry Anthocyanins

Position/ Anthocyanin	3	5	6	7	3'	4'	5'
Cyanidin	OH	OH	H	OH	OH	OH	H
Delphinidin	OH	OH	H	OH	OH	OH	OH
Malvidin	OH	OH	H	OH	OMe	OH	OMe
Peonidin	OH	OH	H	OH	OMe	OH	H
Petunidin	OH	OH	H	OH	OMe	OH	OH

Adapted from Kong *et al.*, 2003
OH: hydroxyl; H: hydrogen; OMe: methyl

Glucose, galactose, arabinose, rhamnose and xylose are the most common sugars linked to the anthocyanidins as mono-, di- or tri-saccharide forms. Among the 17 anthocyanidins found in nature, the six most widely distributed are cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg) and malvidin (Mv) (Prior *et al.*, 2006).

In comparison with other flavonoid compounds anthocyanins show a more complex biochemistry. In aqueous solutions anthocyanins occur in a dynamic equilibrium of four different molecular forms (flavylium cation, quinoidal base, hemiacetal base and chalcone), the amounts of which vary according to solution, pH and structure of the anthocyanins. Anthocyanins exist in the stable flavylium cation form only when the pH < 2. This unique feature is a key factor that affects absorption, bioavailability, metabolism, and therefore the biological activity of anthocyanins (Prior *et al.*, 2006).

The biological effects of anthocyanins are determined by their structural characteristics. Delphinidin and cyanidin, but not pelargonidin, peonidin or malvidin, inhibit lipopolysaccharide-induced COX-2 expression (Hou *et al.*, 2005). The inhibitory actions and the anti-inflammatory properties of delphinidin and cyanidin seem to be related to the *ortho*-dihydroxyphenyl structure of these

anthocyanidins (Hou *et al.*, 2005). Delphinidin and cyanidin, both having a hydroxyl residue at the 3' position, inhibited platelet derived growth factor $_{AB}$ ($PDGF_{AB}$) induced vascular endothelial growth factor (VEGF) expression in the VSMC, but malvidin and peonidin had no effect. In the same study delphinidin and cyanidin directly scavenged ROS and prevented the $PDGF_{AB}$-induced formation of ROS in the VSMC, whereas malvidin and peonidin did not scavenge ROS, but prevented their cellular formation (Oak *et al.*, 2006).

There is great research interest on the structure-activity relationships of flavonoid compounds and flavonoid activity in general, as well as health benefits of flavonoid-rich foods, as flavonoids are one of the most widely distributed groups of plant metabolites and constitute an important part of the human diet (Bravo, 1998).

2.3.2. Dietary Intake of Flavonoids

The primary dietary sources of flavonoids include tea, red wine, fruits and berries, cocoa, chocolate, vegetables and legumes (Manach *et al.*, 2004). Their wide distribution in food sources and the variations in the flavonoid content in a given food, as well as the structural diversity of plant flavonoids complicate the assessment of their dietary intake (Scalbert and Williamson, 2000). Data from Western European studies show that total flavonoid intake varies considerably in western populations, with crude estimates of average intake ranging between 65 to 250mg/day (Erdman *et al.*, 2007). Estimates for individual classes of flavonoid also show great variation among different populations. For instance,

anthocyanidin consumption in Germany is estimated to be 6.5 mg/day (Linseisen *et al.*, 1997). In the US anthocyanidin consumption is estimated to be 1.3 mg/day (Chun *et al.* 2007) and the average proanthocyanidin intake is 58 mg (Gu *et al.*, 2004). The daily intake of anthocyanins in the US, according to the data for anthocyanin concentration and updated food intake from NHANES 2001-2002, is estimated to be 12.5 mg/day/person (Wu *et al.*, 2006).

The average dietary intake of polyphenols in the US was estimated by Kuhnau (1976) to be 1g/day, exceeding the intake of other common antioxidants such as vitamin C (90 mg/day), vitamin E (12 mg/day) and carotenoids (5mg/day). The main sources of polyphenols evaluated for this study were fruits, tea, coffee and wine (Kuhnau, 1976). These data continue to serve as a reference for daily polyphenol intake, even though they are now known to be rather incomplete and inaccurate. Progress has been made in the development of polyphenol profiles of certain foods. However, the accurate determination of intake is not feasible due to lack of a comprehensive food composition database (Erdman *et al.*, 2007).

2.3.3. Flavonoid Absorption, Metabolism and Bioavailability

The rate of absorption and bioavailability of the ingested flavonoids determines their biological functions. The extent to which their metabolism interferes with the antioxidant capacity further dictates their health effects (Hollman and Katan, 1999). In general, bioavailability varies widely among polyphenols, with several factors, such as the dietary source, the food matrix or background diet and the

structure of the compound, explaining the variation of bioavailability among different polyphenolic compounds (Manach *et al.*, 2005).

At the cellular level, the absorption mechanism of a certain flavonoid is determined by its structure. Flavonoids naturally occur mostly in a glycosylated state. The glycosylated flavonoids can be hydrolyzed at the brush border and subsequently the aglycones diffuse across the cell membrane. The glycosylated form may also enter the enterocyte via a sodium-dependent glucose transporter. In the enterocyte, the cytosolic β-glycosidase cleaves the carbohydrate off the flavonoid (Tapiero *et al.*, 2002).

In the intestinal cells, after hydrolysis to the free aglycone, flavonoids are conjugated by methylation, sulfation, glucuronidation or a combination, and bound to albumin for transport to the liver. Further methylation or sulfation of the flavonoid may occur in the liver (Harborne and Williams, 2000). Methylation, sulfation, glucuronidation or a combination are all conjugation reactions that may occur after hydrolysis of a glycosidic flavonoid. The involved metabolic pathways normally follow drug metabolism patterns and are controlled by the distribution and specificity of the catalyzing enzymes. The end result is altered biological properties of the circulating metabolites. After ingestion, flavonoids are quickly eliminated via urine or bile excretion (Scalbert and Williamson, 2000). However, the half-life of conjugated flavonoids is rather long, suggesting that regular flavonoid intake may result in accumulation of these metabolites over time (Young *et al.*, 1999). Microorganisms in the colon can hydrolyze and extensively degrade dietary flavonoids (Kuhnau, 1976). However, some of the anthocyanin

glycosides can be absorbed and excreted unchanged in their glycosidic form (Cao *et al.*, 2001, Mazza *et al.*, 2002; Kahkonen and Heinonen, 2003).

The presence of anthocyanins, rutin and quercetin glycosides was detected in human plasma, confirming that flavonoids can be absorbed in their glycosylated form *in vivo* (Paganga and Rice-Evans, 1997). Oral administration of quercetin resulted in detectable levels of the flavonoid and its derivatives in the plasma and urine of human subjects receiving 200 ml or more of grape juice. In mice, the cumulative amounts of quercetin excreted in the urine after concentrated grape juice administration for four days were 0.7% of the ingested dose (Meng *et al.*, 2004). In pigs, quercetin and quercetin glycosides were shown to be metabolized in the intestinal mucosa. Total bioavailability of quercetin glycoside was higher than the aglycone. However, total bioavailability of quercetin glycoside is dependent on diet composition, as shown by enhanced absorption of the glycoside when meat was incorporated into the animal diet (Cermak *et al.*, 2003). Bioavailability of several flavonoids from almond skins was also investigated in hamsters. All five flavonoids from almond skin (catechin, epicatechin, kaempherol, quercetin and isorhamnetin) were detected in hamster plasma and liver (Chen *et al.*, 2005).

The absorption and bioavailability of the different classes of flavonoids shows a great variation. Most of the research to date suggests that the anthocyanins are the least absorbed flavonoids. However, the low anthocyanin bioavailability observed in various human studies could have been underestimated. On the one hand, some important metabolites might have been ignored or the methods used

for analysis might need to be optimized (Manach *et al.*, 2005). Several studies indicate that anthocyanin absorption starts from the stomach (Ichiyanagi *et al.*, 2004; Ichiyanagi *et al.*, 2006; Felgines *et al.*, 2006). Similar to other flavonoids, the absorption of anthocyanins is greatly affected by the structure of the aglycone and the sugar attached. More free hydroxyl groups and less methoxyl groups can decrease absorption, whereas glucose seems to increase absorption versus galactose (Yi *et al.*, 2006).

The individual anthocyanin content of the dietary source and the form of administration also affect their absorption and bioavailability. A lower rate of anthocyanin excretion after blueberry vs. elderberry consumption was observed in elderly women, although the total amount of anthocyanins consumed was nearly the same (Wu *et al.*, 2002). The administrated dose is another factor to consider in anthocyanin bioavailability. The 24 h excretion of red cabbage anthocyanins increased with higher dose, whereas the urinary recovery of intact anthocyanins decreased, also in a dose-dependent manner (Charron *et al.*, 2007). Metabolic conversion in the liver or kidney accounts for the difference between anthocyanin profiles in these organs and blood plasma, after bilberry extract administration in rats (Ichiyanagi *et al.*, 2006).

Anthocyanins that are not absorbed in the stomach or the small intestine may be transferred to the colon, whereas, absorbed anthocyanins can also reach the colon through bile excretion. Extensive degradation and hydrolysis of anthocyanins by the intestinal microflora has been shown *in vitro* (Aura *et al.*, 2005; Keppler *et al.*, 2005). The anthocyanin metabolites produced by the

intestinal microflora are likely to contribute to the biological effects of the anthocyanins, but they are often overlooked (Prior *et al.*, 2006). For instance, in a study by Tsuda *et al.*, (1999) it was proposed that cyanidin-3-O-β-D-glucoside (C3G) administered orally in rats is first hydrolyzed in the intestine by β-glucosidase and the aglycone part produced (cyanidin) is degraded to protocatechuic acid, which may have a higher antioxidant activity (Tsuda *et al.*, 1999). In a recent study in humans, protocatechuic acid was found to be the major metabolite of cyanidin glycosides from blood orange juice, accounting for approximately 73% of the ingested dose. Hence, it was proposed that protocatechuic acid may be responsible for the health effects attributed to cyanidin intake (Vitaglione *et al.*, 2007).

It is also noteworthy that blueberry and blackberry anthocyanins can cross the blood-brain barrier. Anthocyanins from blueberry supplementation were detected in different brain regions related to learning and memory (Andres-Lacueva *et al.*, 2005), while both native and methylated anthocyanins from blackberry were shown to be transported from the blood to the brain tissue (Talavera *et al.*, 2005). A profound knowledge and understanding of the absorption, metabolism and bioavailability of anthocyanins and flavonoids in general can substantially contribute to clarify the role these bioactive compounds play in health and disease. Additional research studies relating the results of *in vitro* studies with the actual *in vivo* effects and acclaimed health benefits of flavonoids are needed.

In general, flavonoids are known to protect health in several ways due to their antioxidant, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities (Middleton *et al.*, 2000).

Most flavonoid-related health claims are based on their antioxidant properties. This is also the case for cardiovascular health, since oxidative stress is an important determinant of CVD.

2.3.4. Flavonoids and Cardiovascular Health

Oxidative stress induced by ROS plays a causative role in various CVDs such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure (Kukreja and Hess, 1992).

In the SHR, long term consumption of an antioxidant diet enriched with vitamin E and C, zinc and selenium, was shown to reduce oxidative stress and improve hypertension (Rodriguez-Iturbe *et al.*, 2003). Additionally, antioxidant treatment with vitamins E and C was shown to reverse the impaired endothelium-dependent vascular relaxation in the SHR. The effects of these antioxidant vitamins were associated with enhanced eNOS activity and increased NO generation, as well as reduced NADPH activity and O_2^- production (Ulker *et al.*, 2003). However, the existing scientific evidence does not justify routine use of antioxidant supplements for prevention or treatment of CVD in humans, as more research is needed to clarify the discrepancies between randomized clinical trials and population studies. Instead of antioxidant supplementation, consumption of food sources such as fruits, vegetables, whole grains and nuts that are rich in

antioxidants and other cardioprotective nutrients is recommended (Kris-Etherton *et al.*, 2004).

The Women's Health Study revealed a lower risk of CVD, and especially, myocardial infarction, for women with higher fruit and vegetable consumption (Liu *et al.*, 2000). A high fruit, berry and vegetable intake was also associated with reduced CVD risk factors and overall mortality in men (Rissanen *et al.*, 2003). Therefore, increased fruit and vegetable consumption is considered a primary preventive measure against CVD. Many commonly consumed fruits and vegetables, as well as grains, herbal products and beverages, contain significant amounts of phenolic compounds. The relationship between foods rich in flavonoids, such as tea, berries, cocoa, chocolate and wine, and CVD has been examined by epidemiological and experimental studies that overall suggest a protective role of flavonoids (Erdman *et al.*, 2007). The association between flavonoid intake and CHD assessed in a prospective cohort study revealed a much lower cardiovascular risk in individuals with the highest flavonoid intake (Hertog *et al.*, 1993). Furthermore, Finish women and men with lower flavonoid consumption over a 20-year period were shown to have higher CHD risk (Knekt *et al.*, 1996). Flavonoids demonstrate protective effects against the initiation and progression of atherosclerosis. The "French paradox" originally described in 1979 (Renaud and de Lorgeril, 1992), resulted in many studies of grape flavonoids, followed later on by studies of tea, chocolate or pomegranate (Erdman *et al.*, 2007). The "paradox" was that the French had a much lower CHD mortality rate, despite a higher consumption of saturated fat. Hence, it was postulated that the

daily consumption of red wine with meals provides cardiovascular protection. The flavonoids from red wine, particularly flavan-3-ols, anthocyanins, flavonols and proanthocyanidins, and not solely the alcohol, are now considered the primary protective components (Erdman *et al.*, 2007). Red wine polyphenols are shown to protect against oxidative stress, platelet aggregation and thrombogenesis. With regard to blood vessels, these compounds are powerful vasodilators and play an important role in preservation of endothelium integrity by inhibiting endothelial and muscle cell proliferation and migration, and angiogenesis processes (Cordova *et al.*, 2005).

Other foods or juices rich in polyphenols have also been shown to have beneficial effect on various factors related to CVD including LDL-oxidation, platelet activity or inflammation (Stein *et al.*, 1999; Aviram *et al.*, 2004; Demrow *et al.*, 1995; Pearson *et al.*, 1999; Fuhrman *et al.*, 2005; Freedman *et al.*, 2001; Iijima *et al.*, 2002; Holt *et al.*, 2006; Murphy *et al.*, 2003; Youdim *et al.*, 2000; Youdim *et al.*, 2002; Bagchi *et al.*, 2004). Short term ingestion of purple grape juice, which contains flavan-3-ols, flavonols, anthocyanins and proanthocyanidins, improved endothelial function and reduced LDL susceptibility oxidation in patients with established CAD (Stein *et al.*, 1999). Similarly, pomegranate juice, which is rich in specific flavonoids such as punicalagin and anthocyanins, was shown to have a protective effect against the atherosclerotic process. Long term pomegranate juice intake by patients with carotid artery stenosis reduced LDL oxidation, but also BP and intima-media thickness of the carotid artery; an effect attributed to the potent antioxidant properties of the juice

flavonoids (Aviram *et al.*, 2004). Red wine was shown to inhibit *in vivo* platelet-mediated experimental coronary thrombosis, whereas equal amounts of pure alcohol or alcohol-free grape juice had no effect (Demrow *et al.*, 1995). Apple juice was shown to inhibit *in vitro* copper catalyzed human LDL oxidation (Pearson *et al.*, 1999). Incubation of macrophages with pomegranate juice caused a significant reduction in cellular uptake of oxidized LDL and cholesterol biosynthesis, as well as lower levels of cellular oxidative stress (Fuhrman *et al.*, 2005). Oral supplementation by healthy volunteers for two weeks, and *in vitro* incubation of platelets with purple grape juice, were shown to decrease platelet aggregation and superoxide production and to increase platelet-derived NO release. The observed effect was dose-dependent, related to partial inhibition of protein kinase C stimulation and attributed to antioxidant and/or a direct effect of selected flavonoids (Freedman *et al.*, 2001). Grape flavonoids can also inhibit the abnormal proliferation of VSMCs, a process involved in atheroma development and intimal thickening. Additionally, platelet-derived growth factor-BB (PDGF-BB) and the subsequent migration of VSMC was inhibited by grape flavonoids through inactivation of the phosphatidylinositol 3-kinase (PI3K) and p38 mitogen activated protein kinase (MAPK) pathway (Iijima *et al.*, 2002). The consumption of flavonoid-rich cocoa and chocolate has been associated with a reduction in platelet activity (Holt *et al.*, 2006). Supplementation for 28 days with cocoa flavanols and related procyanidin oligomers significantly increased plasma epicatechin and catechin concentrations and decreased *in vivo* human platelet activation and aggregation (Murphy *et al.*, 2003). Incubation of red blood cells

with blueberry anthocyanins was found to significantly enhance cell resistance to ROS production (Youdim *et al.*, 2000). The protective effect of anthocyanins against ROS was also observed *in vivo* after oral supplementation in rats (Youdim *et al.*, 2000). Blueberry and cranberry polyphenols were able to enter endothelial cells and thereby reduce cell vulnerability to increased oxidative stress at both the membrane and cytosol level (Youdim *et al.*, 2002). A reduction of tumor necrosis factor-α (TNF-α)-induced upregulation of inflammatory mediators, such as interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1), was also observed in this *in vitro* study (Youdim *et al.*, 2002). These inflammatory mediators are involved in the recruitment of leukocytes to sites of damage or inflammation along the endothelium (Youdim *et al.*, 2002). An anthocyanin-rich berry extract cocktail from wild blueberry, bilberry, cranberry, elderberry, raspberry seeds and strawberry tested *in vitro* was shown to possess antioxidant, antiangiogenic and anticarcinogenic properties (Bagchi *et al.*, 2004).

Individual flavonoids appear to act upon several enzymatic systems related to the development of CVD in a fashion presumably dictated by their structural characteristics (Gryglewski *et al.*, 1987; Loke *et al.*, 2008; Chung *et al.*, 1993; Hou *et al.*, 2005; Lamy *et al.*, 2006; Oak *et al.*, 2006; Adhikari *et al.*, 2005; Yan *et al.*, 2002; Serraino *et al.*, 2003; Rechner and Croner, 2005). The flavonols, quercetin and rutin, as well as the flavanes, cyanidol and meciadonol, inhibited platelet lipoxygenase activity, but only quercetin and rutin inhibited cyclooxygenase activity. The two flavonols but not the two flavanes, dispersed

platelet thrombi adherent to the rabbit aortic endothelium and prevented platelet aggregation (Gryglewski *et al.*, 1987). At least two of the major *in vivo* metabolites of quercetin were shown to retain significant inhibitory activity of pro-inflammatory eicosanoids such as PGE_2 and leukotriene B_4 (LTB_4) derived from the COX and lipoxygenase enzymatic pathways respectively (Loke *et al.*, 2008). Additionally, quercetin and related compounds were shown to inhibit platelet aggregation, while quercetin also showed vasorelaxant action in the thoracic rat aorta (Chung *et al.*, 1993). In a recent study, delphinidin and cyanidin, but not pelargonidin, peonidin or malvidin, inhibited lipopolysacharide-induced COX-2 expression. The *ortho*-dihydroxyphenyl structure of anthocyanidins seems to be related to the inhibitory actions and the anti-inflammatory properties. Furthermore, delphinidin, the most potent inhibitor among the anthocyanidins tested, suppressed COX-2 by blocking several MAPK-mediated pathways (Hou *et al.*, 2005). Delphinidin was also found to be the most potent inhibitor of vascular endothelial growth factor (VEGF) receptor phosphorylation *in vitro* and *in vivo* among six isolated anthocyanins (cyanidin, delphinidin, pelargonidin, peonidin and petunidin) (Lamy *et al.*, 2006). Delphinidin and cyanidin, both having a hydroxyl residue at the 3' position, inhibited platelet-derived growth factor $_{AB}$ $^{-}$ ($PDGF_{AB}$) induced VEGF expression by preventing activation of p38 MAPK and C-Jun N-terminal kinase (JNK) in the VSMC. In the same study, red wine polyphenols, delphinidin and cyanidin directly scavenged ROS and prevented the $PDGF_{AB}$ -induced formation of ROS in the VSMC, whereas malvidin and peonidin did not scavenge ROS, but prevented their cellular

formation (Oak *et al.*, 2006). Cyanidin, cyanidin 3-galactoside and cyanidin 3-glucoside from Amelanchier fruits were shown to inhibit *in vitro* COX-1 and -2, as well as lipid peroxidation in a dose-dependent manner (Adhikari *et al.*, 2005). Cyanidin-3 galactoside and several quercetin glycosides isolated from cranberry were found to possess comparable antioxidant activity to vitamin E. However, the capacity of cyanidin-3-galactoside and free quercetin to prevent Cu^{2+} catalyzed LDL oxidation was superior to vitamin E (Yan *et al.*, 2002). Cyanidin-3-O-glucoside from blackberry juice had a protective *in vitro* effect against endothelial dysfunction and vascular failure, induced by peroxynitrite. By scavenging the free radical, cyanidin-3-O-glucoside reduced the peroxynitrite-induced suppression of mitochondrial respiration, DNA damage and the nuclear enzyme poly (ADP-ribose) synthase (PARS) activation in HUVECs. Energy depletion and cellular injury is the end result of massive ADP-ribosylation of nuclear proteins by PARS (Serraino *et al.*, 2003). Anthocyanins such as delphinidin and cyanidin as well as various colonic metabolites of a representative phenolic mixture were also shown to inhibit *in vitro* platelet function (Rechner and Croner, 2005).

The research evidence for the cardioprotective effects of flavonoid-rich foods as well as their individual bioactive compounds is mounting and promising, so is the evidence for the benefits of flavonoids on vasomotor function more specifically.

2.3.5. Flavonoids and Vasomotor Function

Flavonoid-rich foods and isolated flavonoids have been shown to have a positive effect on endothelium-dependent vasodilation and BP in both *in vitro* and *in vivo*

studies. A series of dietary *ex vivo* studies on the wild blueberry fruit conducted in Dr. Klimis-Zacas' laboratory have shown the potential of wild blueberries in protecting the endothelial function (Norton *et al.*, 2005; Kalea *et al.*, 2005; Clark, 2007). According to Norton *et al.*, wild blueberries incorporated into the diet affected the vascular smooth muscle contractile machinery by suppressing the α-1 adrenergic receptor-agonist-mediated contraction through an endothelium-dependent pathway (Norton *et al.*, 2005). Furthermore, wild blueberry consumption altered the structure of the extracellular matrix of Sprague Dawley (SD) rat aortas, by increasing the concentration of glycosaminoglycans (GAGs) and decreasing the sulfation of all GAG type molecules, suggesting a possible effect of blueberries on endothelial and vascular smooth muscle signal transduction pathways (Kalea *et al.*, 2005). Results of a most recent study documented that wild blueberries appeared to affect the endothelium-dependent vasodilation of the aorta by modulating cell membrane-agonist interactions in response to Ach in young normotensive SD rats and SHRs. Wild blueberries affected the endothelial-dependent vasodilation in young SHR aorta most likely, by modulating a key pathway of endothelial function, the cyclooxygenase (COX) pathway (Clark 2007).

To our knowledge, the above studies are the only ones that investigate the dietary effect of wild blueberries on endothelium-dependent vasodilation *ex vivo*. Numerous studies have been conducted on other flavonoid–rich foods or isolated compounds and have indicated the potential role of flavonoids in maintenance and improvement of vasomotor tone. Short-term ingestion of purple grape juice

improved flow mediated vasodilation and reduced LDL susceptibility to oxidation in CAD patients (Stein *et al.*, 1999). The beneficial effect of purple grape juice on endothelium function was confirmed by another study in CAD patients (Chou *et al.*, 2001). Consumption of flavonol-rich dark chocolate decreased blood pressure and improved endothelium-dependent relaxation in patients with essential hypertension. Furthermore, insulin resistance and serum LDL cholesterol were reduced (Grassi *et al.*, 2005). Moreover, short term intervention with flavonoid-rich chocolate increased plasma epicatechin concentration and improved endothelium-dependent vasodilation in healthy adults independent of changes in oxidative stress and lipid profiles (Engler *et al.*, 2004). When patients with at least one cardiovascular risk factor were administered a single dose of cocoa drink rich in flavan-3-ols, NO bioactivity was transiently increased over a 2 hour period. Endothelial vasodilation, measured by brachial flow mediated dilation (FMD), was also increased (Heiss *et al.*, 2003). Short and long-term black tea consumption could reverse endothelial dysfunction in CAD patients as reflected by increased flow-mediated dilation (Duffy *et al.*, 2001). Similarly, in healthy subjects endothelium-dependent vasodilation was significantly and consistently increased by regular tea consumption (Hodgson *et al.*, 2002). A recent study suggested that the flavanol compound epicatechin, mediates, at least in part, the beneficial vascular effects associated with the consumption of flavanol-rich cocoa in humans, based on the finding that pure epicatechin ingested by humans closely and quantitatively mimicked the vascular effects of flavanol-rich cocoa (Schroeter *et al.*, 2006).

In SD rats, a diet rich in dealcoholated red wine, quercetin or catechin induced endothelium dependent vasodilation via the NO-cGMP pathway (Benito *et al.*, 2002). Black currant concentrate, consisted of 10.83% anthocyanins, mostly delphinidin and cyanidin, induced endothelium-dependent vasodilation and NO release in SD rat aorta. It was suggested that the histamine receptor H_1 may be involved in the process (Nakamura *et al.*, 2002). Extract of wine phenolics was shown to reduce the elevation of blood pressure in Stroke-Prone Spontaneously Hypertensive Rat (SHRSP), presumably through the observed increase in the vasorelaxation activity (Mizutani *et al.*, 1999). Incubation of femoral arterial rings of Wistar Kyoto rats with red wine polyphenol powder, particularly rich in proanthocyanidins, increased NO synthase activity and thereby, vasorelaxation (Zenebe *et al.*, 2003). Anthocyanin-enhanced extracts from chokeberry and bilberry, but not elderberry produced dose-dependent vasorelaxation of porcine coronary arteries. Even low concentrations of anthocyanins showed a significant capacity to prevent loss of endothelium-dependent vasodilation due to exogenous ROS exposure (Bell and Gochenaur, 2006). The isolated flavonoids baicalein, flavone and quercetin administered in SHRs for four weeks significantly decreased endothelium-dependent vasodilation in response to Ach (Machha *et al.*, 2005). Acute exposure of the SHR aorta to quercetin (20 min incubation with 10 μmol/L) was shown to improve endothelium-dependent relaxation and reduce the α_1-adrenergic receptor-mediated contractile response with potency greater than vitamin C (Ajay *et al.*, 2006). Daily quercetin oral administration (10 mg/kg) for five weeks reduced significantly systolic and

diastolic blood pressure and elicited functional vascular changes in the SHR, but not in the WK rat. Reduced cardiac and renal hypertrophy were also observed in the SHR after the long term exposure to quercetin (Duarte *et al.*, 2001). In addition to lowering BP and heart rate of SHR, and enhancing endothelium-dependent vasodilation, quercetin enhanced eNOS activity and decreased NADPH oxidase-mediated superoxide generation (Sanchez *et al.*, 2006). Delphinidin, but not malvidin or cyanidin, elicited endothelium-dependent vasodilation in WK rat aorta. The vasodilatory effect was completely mediated by NO activity. The results indicated that, among anthocyanins, specific structures are needed to modulate endothelium-dependent relaxation (Andriambeloson *et al.*, 1998). Red wine flavonoids were shown to up-regulate eNOS expression, increase NO production *in vitro,* and therefore, improve endothelial dysfunction (Diebolt *et al.*, 2001). Wine, grape juice and grape skin extracts were shown to produce endothelium-dependent relaxation *in vitro*, which was mediated by the NO-cGMP pathway (Fitzpatrick *et al.*, 1993). Several plant extracts also caused endothelium-dependent vasodilation through an increase in cGMP levels (Fitzptrick *et al.*, 1995). Long term incubation of HUVECs with the crude extract or organic subfraction of artichoke leafs, rich in flavonoids, increased eNOS expression and NO production. Additionally ex *vivo* incubation of aortic rings with the organic subfraction of artichoke leafs enhanced the NO mediated vasodilator response to Ach (Li *et al.*, 2004). Anthocyanin-rich berry extracts showed considerable inhibitory effects on NO production from macrophages, and their inhibitory effects were significantly correlated with the content of total phenolics,

tartaric ester, flavonols, and anthocyanins (Wang and Mazza, 2002). The flavonols quercetin and myricetin, and the anthocyanins/anthocyanidins pelargonidin, cyanidin, delphinidin, peonidin, malvidin, malvidin 3-glucoside, and malvidin 3,5-diglucosides, demonstrated >50% inhibition on NO production without affecting cell viability (Wang and Mazza, 2002). Cyanidin and delphinidin were both shown to significantly decrease ET-1 production and increase eNOS activity in HUVECs. Delphinidin activity upon eNOS increase was dose-dependent and greater in comparison with cyanidin (Lazze *et al.*, 2006). The superior vasoprotective effect of delphinidin was positively correlated with the greater antioxidant activity due to the presence of three hydroxyl groups in the B-ring (Lazze *et al.*, 2006). Cyanidin-3-glucoside induced eNOS expression and NO release in bovine vascular endothelial cells (Xu *et al.*, 2004a). The same research team suggested that cyanidin-3-glycoside can regulate phosphorylation of eNOS and the protein kinase Akt, which induces NO release via eNOS activation. Cyandin-3-glucoside also affects the interaction of eNOS with sGC increasing the cGMP production and subsequently inducing vasorelaxation (Xu *et al.*, 2004b). The inhibition of protein kinase C and cAMP release was proposed by Duarte *et al.* (1993) as the main vasodilatory mechanism of flavonoids. The potency of the flavonoids to induce vasorelaxation correlated with the potency to inhibit protein kinase C. Inhibition of cyclic nucleotide phosphodiesterase or decreased Ca^{2+} may also contribute to the vasodilatory effect. Additionally, the structure seems to determine flavonoid activity with the flavonols quercetin,

kaempherol and 5-O-methyl-quercetin being the most potent, followed by flavones and lastly flavanols (Duarte *et al.*, 1993).

The studies discussed above, suggest a great potential of flavonoids and anthocyanins in particular, for improvement of endothelial function. However, the exact molecular and biochemical mechanisms of action of these bioactive substances remain to be elucidated. With regard to the cardiovascular health effect of wild blueberries, human or animal studies are limited. Moreover, the vast majority of the research conducted thus far, was aimed at studying the polyphenolic extracts of blueberries rather than the whole fruit added to the diet. In addition, research on the blueberry extracts has been conducted mainly *in vitro* (Bagchi *et al.*, 2004; Youdim *et al.*, 2000; Youdim *et al.*, 2002).

In the present dietary study, the potential role of wild blueberries and their possible mechanism of action on endothelium-dependent vasodilation in response to Ach were investigated in SHR and normotensive WK rats in an *ex vivo* experimental setting.

CHAPTER 3

METHODS AND MATERIALS

3.1. Animal Models

Twenty male young adult Spontaneously Hypertensive rats (SHR) were used as a model of endothelial dysfunction and 20 male young adult Wistar Kyoto rats (WK) were used as a model of functional endothelium (controls). The Animal Care and Use Committee of the University of Maine approved the animal care and the experimental procedures.

Numerous pharmacological (Luscher and Vanhoutte, 1986; Xiao and Pang, 1994; Yang *et al.*, 2002) and dietary studies (Duarte *et al.*, 2001; Machha *et al.*, 2005; Rodriguez-Iturbe *et al.*, 2003) have utilized the SHR as a model of endothelial dysfunction. The normotensive WK, presenting a functional endothelium, is used as a control to the SHR, since SHR as a strain was developed from outbred WK rats (Okamoto and Aoki, 1963).

Spontaneously Hypertensive rats were purchased from Charles River Laboratories (Wilmington, MA), while the WK rats were purchased from Taconic Farm (Hudson, NY). All rats were purchased at the age of 12 weeks, placed on dietary treatments for nine weeks and sacrificed at the age of 21 weeks.

The age of 10 weeks in the SHR is considered as the early hypertensive stage, with a systolic BP at approximately 170 mmHg (Tenase *et al.*, 1982). Spontaneously hypertensive rats are expected to develop hypertension at the age of 7 to 15 weeks and their systolic BP has been shown to plateau at

approximately 200 mmHg (Yamori, 1984). Therefore, by 21 weeks of age SHRs have developed full-blown hypertension.

The animals were housed in the Small Animal Facility at the University of Maine in individual stainless-steel mesh-bottomed cages in an environmentally controlled room maintained at 22°C with a 12:12-hour light: dark cycle. In order to avoid possible infection, each strain was housed in separate rooms. All animals were weighed weekly to determine possible differences in growth rate within treatment groups. Also food consumption was measured daily to determine possible differences in food intake among diet groups. Tap water and food was provided *ad libitum*.

For the **functional arterial property experiments**, rats from each strain, SHR and WK, were randomly assigned to one of two diets: control diet (C) and blueberry diet (B) (control + 8% w/w wild blueberry powder substituting for dextrose) for a period of nine weeks (Norton *et al.*, 2005, Kalea *et al.*, 2006, Clark 2006). Similarly, for the **blood pressure measurement experiments**, rats from each strain, SHR and WK, were randomly assigned to one of the two diets: C and B, as mentioned above, for a period of nine weeks. The C diet groups were SHR-C and WK-C, n=10 each, and the B diet groups were SHR-B and WK-B, n=10 each.

3.2. Animal Diet Composition

The animal diets were prepared in our lab, stored at 4°C and used within 5 to 7 days. The purified diet ingredients used were dextrose, egg white solids, vitamin

mix, D,L-methionine, biotin, mineral mix, and corn oil (Table 3.1). The mineral mix was purchased from ICN Biomedicals (Cleveland, OH), whereas all the other ingredients were purchased from Harlan Teklad (Madison, WI). For the **functional arterial property experiments**, a wild blueberry composite was provided by Wayman's (Cherryfiled, ME) and was freeze-dried with standard procedures by Oregon Freeze Dry (Albany, OR). For the **blood pressure measurement experiments**, the freeze-dried wild blueberry powder 1.5% N11 from Van Drunen Farms (Momence, IL) was utilized instead of the composite due to lack of availability. The N11 wild blueberry powder from Van Drunen Farms is standardized to contain a minimum 1.5% of total anthocyanins. For both experiments, the wild blueberry powder was provided as 8% (w/w) of total diet content, which is approximately equivalent to daily human consumption of a half cup of fresh wild blueberries (Norton *et al.*, 2005). The diet composition is presented in Table 3.1.

Table 3.1. Diet Composition

Dietary Component	Control Diet (g)	Blueberry Diet (g)
Dextrose	691	611
Wild Blueberry	0	80
Egg white solids	200	200
Mineral mix (1 g/kg Mn)	35	35
Vitamin mix	10	10
Biotin	0.002	0.002
D-L-Methionine	4	4
Corn oil	60	60
(Total weight)	1000	1000

3.3. Drugs and Solutions

The chemicals for the Physiologic Salt Solution (PSS), as well as the drugs used, acetylcholine chloride (Ach), L-Phenylephrine (Phe), L-N^G-monomethyl arginine (L-NMMA), mefenamic acid (MFA) and pentobarbital sodium salt, were purchased from Sigma Aldrich (St. Louis, MO). The heparin sodium injection USP (1000 USP units/ml), was purchased from Baxter (Deerfiled, IL). The PSS composition was the following: NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 12.5 mM and dextrose 11.1 mM.

3.4. Aortic Ring Preparation

The use of animal arterial rings is an established experimental approach for studying the role of the endothelium on arterial functional properties. Findings from animal ring studies agree with those from isolated human vessels (Vanhoutte, 1999).

Rats were anesthetized with 95%CO_2/ 5%O_2 for approximately 2 min. Blood samples were collected via cardiac puncture. The thoracic aorta was excised and placed in a silicon–coated petri dish, filled with PSS and cleaned from the surrounding connective tissues and blood clots. The middle part of the aorta was divided into four rings, each 3 mm of length, with surgical scissors (George Tiemann & Co. Hauppauge, NY). The shape, length, or any damage of the aorta during ring preparation were documented.

Each aortic ring was suspended by two stainless steel wire triangles and mounted in a Radnoti tissue bath (Radnoti Glass Technology Inc. Monrovia, CA),

containing PSS at 37°C and aerated with a gas mix of 95%O_2/ 5%CO_2 (pH 7.45). The aortic rings were connected to Tissue Force Analyzers (TFA) (model 410, Micro-Med Louisville, KY), that measured the force developed in the aorta in response to the different drugs added in the tissue bath. The TFAs were connected to a computer system that recorded via Digimed software, DMSI-210 (Micro-Med Louisville, KY) the force developed in the aorta, which as used to estimate the experimental parameters, maximum force of vessel relaxation (Fmax) and vessel reactivity (pD_2). All rings from each animal were mounted in the tissue baths within 60 min from the administration of anesthesia.

3.5. Experimental Design

3.5.1. Physiological Assessment of Arterial Functional Properties

Preliminary experiments were conducted to determine the Phe dose for the maximal contraction of the aortic rings for each of the two strains by constructing Phe dose-response curves. Following preconditioning of rings with Ach (10^{-8}M) and Phe (10^{-8}M) for a period of 10 min, ten cumulative Phe doses were utilized (10^{-9}, 3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6}, 10^{-5}, 3×10^{-5}M) for constructing a Phe dose-response curve. A drug-tissue contact-time of six min was allowed for each Phe dose to achieve maximum contraction. The Phe dose that resulted in the maximal contraction of arterial rings was found in both strains to be 10^{-6}M.

3.5.1.1. Vasodilation in the Absence of Inhibitors

Following a 45 min equilibration period under 1.5 g preload, rings were preconditioned with Ach (10^{-8}M) and Phe (10^{-8}M) for a period of 10 min and then washed out with PSS. The rings were then precontracted with one maximal dose of the α_1-adrenergic agonist Phe (10^{-6}M). All the rings reached plateau of maximal contraction within 10 min. Following the 10-min Phe precontraction, eight concentrations of Ach (10^{-9}, 3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6}M) were applied in order to construct the dose-response curve. A drug-tissue contact-time of 6 min was allowed for each Ach concentration to achieve the maximum relaxation to the initial precontraction (Figure 3.2).

3.5.1.2. Vasodilation in the Presence of Inhibitors

Inhibitors used were: L-NMMA (10^{-4}M) (NOS inhibitor) and MFA (10^{-5}M) (non-selective COX inhibitor). Inhibitors were added after washing with PSS and were allowed to stay in the tissue bath for 25 min before adding the Phe precontraction dose (10^{-6}M). Two separate Ach dose-response curves were generated. For the first Ach dose-response curve, two of the rings were challenged with MFA (COX-pathway inhibition), while no inhibitor was added in the other two aortic rings. For the second Ach dose-response curve, L-NMMA only was added in two of the rings (NOS-pathway inhibition), while both L-NMMA and MFA were added to the other two aortic rings (COX and NOS-pathway inhibition). Thus, the effect of the inhibitors on the NO and COX pathways was studied separately and simultaneously (Figure 3.1, 3.2).

Figure 3.1. Vascular Ring Study Experimental Design

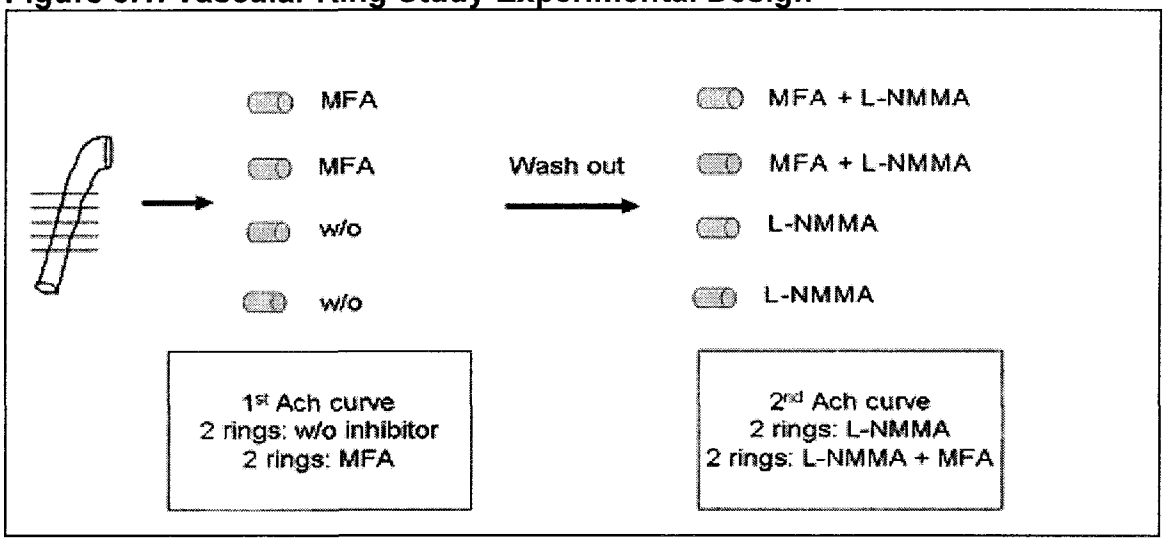
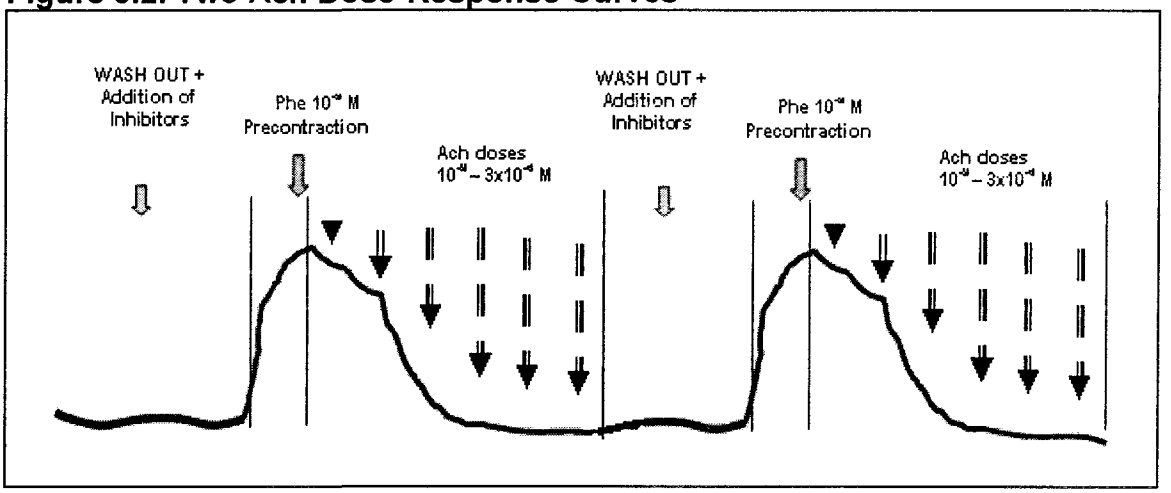


Figure 3.2. Two Ach Dose-Response Curves



The digitized raw data were used to generate individual dose-response curves and the following experimental parameters: maximal force of vessel relaxation (Fmax), dose that inhibits 50% of vessel response (EC_{50}) and vessel reactivity (pD_2). The force of relaxation at each Ach dose was determined as the percent relaxation of the initial precontraction and used to construct dose-response curves for each treatment. Among the values of vasorelaxation force at each of

the eight Ach doses, the highest from each treatment group, was picked as the Fmax. The EC_{50} values were obtained by transforming the dose response curve to semi-log curves. Finally, the pD_2 values were calculated as the - log EC_{50} in order to give normally distributed data and used as an index of receptor agonist interaction (Beach *et al.*, 2001).

Table 3.2. Experimental Parameters

Experimental Parameter	Biological Interpretation	Assessment Method
Fmax	Maximal force of vessel relaxation	Dose-response curve
EC_{50}	Dose to inhibit 50% of vessel response	Transforming dose-response curves to semi log curves
pD_2	Vessel reactivity (receptor-agonist interaction)	Negative log EC_{50} to give normally distributed data

3.5.2 Blood Pressure Measurement

After the nine week period of dietary treatment, rats were anesthetized with sodium pentobarbital solution (60 mg/ml saline), (60mg/kg body weight) intraperitoneally. A blunt dissection technique was used to dissect the neck area of the animal, identify the carotid bundle and separate the left carotid artery from nerve and muscle tissue (Whitesall *et al.*, 2004). The left carotid artery was cannulated with polyethylene tubing (PE-50) filled with heparinized saline (100 USP units/ 5 ml saline) and connected to a CyQ 103 pressure transducer (Cybersense Inc. Nicholasville, KY) for the recording of systolic, diastolic and mean arterial BP on a CyQ 302 recorder (Cybersense Inc. Nicholasville, KY). Values for systolic, diastolic and mean arterial BP were recorded over a period of 30 min after connecting the animal to the transducer. After BP data were

obtained, blood was collected through the cannula, centrifuged at 2500 rpm for 10 min and stored at - 80°C for further analysis.

3.6. Statistical Analysis

A Student t-test was used to determine the possible effect of diet on rat body weights and food consumption between the diet groups within each strain (WK-B vs. WK-C and SHR-B vs. SHR-C).

The Fmax and pD_2 values in the absence or presence of inhibitors were compared between treatment groups to determine the possible effect of diet. Two-way Analysis of Variance (ANOVA) was used to compare equal number of rank ordered observations for the Fmax and pD_2 measurements. Two-way ANOVA, was used to determine the possible effect of diet on systolic, diastolic and mean BP between the diet groups within each strain (WK-B vs. WK-C and SHR-B vs. SHR-C), as well as to determine the differences in blood pressure between strains within each treatment group (WK-C vs. SHR-C and WK-B vs. SHR-B).

The Sigmastat Statistical Program Package (SAS Institute Cary, NC) was used to perform the statistical analysis. All values were given as mean ± SEM (standard error of mean); differences were considered statistically significant at $p \leq 0.05$.

CHAPTER 4

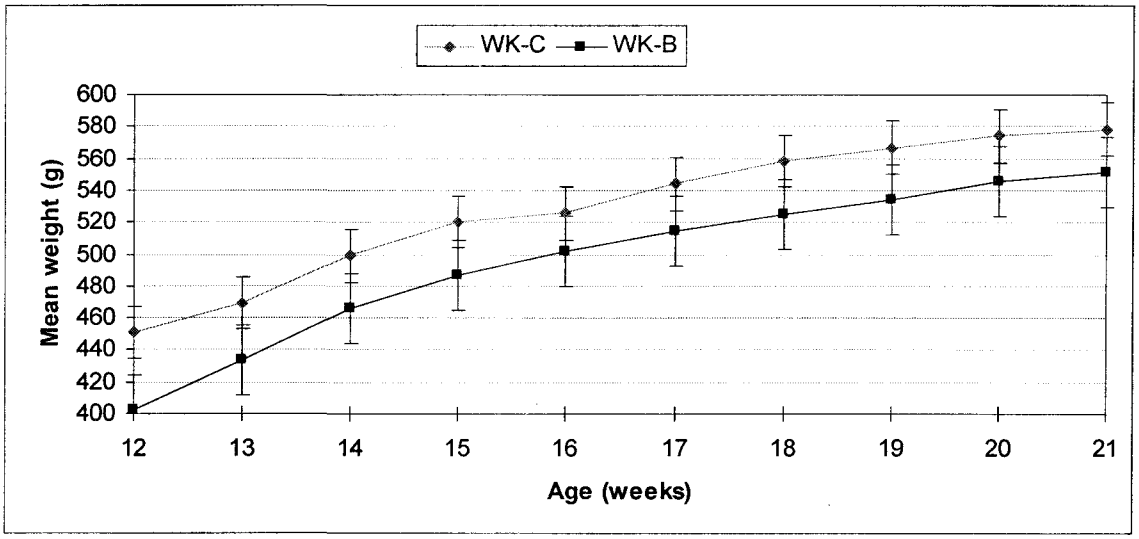
RESULTS

4. 1. Wistar Kyoto Rats

4.1.1. Rat Growth and Weight

Figure 4.1 represents the growth rate of WK rats fed control (WK-C) and wild blueberry-enriched (WK-B) diet from 12 to 21 weeks of age. The rate of growth during the nine week time-period was not significantly different between the two diet groups. The final mean body weights at the end of the diet study were 576 ± 16.50 g and 552 ± 22.04 g for the WK-C and WK-B group respectively, which were not significantly different, ($p = 0.39$). Additionally, no statistically significant difference was found in the food intake of the two diet groups, 26 ± 0.63 g in the WK-C group and 26 ± 0.63 g in the WK-B group, ($p = 0.58$).

Figure 4.1. Growth Rate of WK Rats (Weekly Weight*)



* Mean ± SEM (g)
WK-C: control group, (n = 10); WK-B: blueberry group, (n = 10)

4.1.2. Effect of Diet on Maximum Vasodilation Force (Fmax)

The effect of diet on the Fmax in response to Ach a) in the absence of inhibitors or in the presence of b) the COX-pathway inhibitor mefenamic acid (MFA) or c) the NO-pathway inhibitor L-N^G-monomethyl arginine (L-NMMA) or d) both inhibitors added simultaneously is presented in Table 4.1 and Figure 4.2. No significant difference was found between diet groups for any of the drug treatments. The Fmax in response to Ach observed in the WK-B group (97.40 ± 1.78), was not significantly different from the Fmax observed in WK-C group (97.92 ± 1.78), ($p = 0.84$). The Fmax observed in the WK-B group in the presence of MFA (98.44 ± 1.20) tended to be higher than in the WK-C group (95.60 ± 1.20), but the difference was not statistically significant, ($p = 0.13$). In the WK-B group in the presence of L-NMMA (49.41 ± 1.91), Fmax was not significantly different from the WK-C (50.36 ± 1.91) group, ($p = 0.73$). Similarly, in the presence of both inhibitors Fmax was not significantly different between the two diet groups (WK-B: 46.91 ± 1.84 vs. WK-C: 43.70 ± 1.84), ($p = 0.25$).

Table 4.1. Effect of Diet on the Fmax* in the Absence or Presence of Inhibitors in the WK Rats

Diet group	Fmax Ach	Fmax Ach MFA	Fmax Ach L-NMMA	Fmax Ach MFA + L-NMMA
WK-C	97.92 ± 1.78	95.60 ± 1.20[b]	50.36 ± 1.91[b]	43.70 ± 1.84[b]
WK-B	97.40 ± 1.78	98.44 ± 1.20	49.41 ± 1.91[b]	46.91 ± 1.84[b]

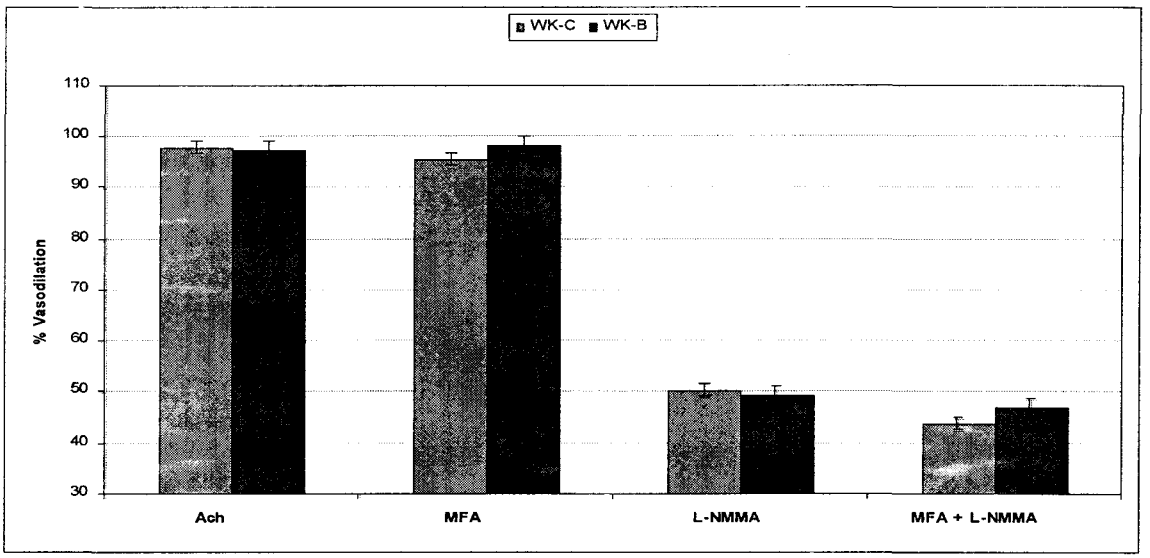
* Mean ± SEM

[b] Statistically significant compared to Ach treatment within the same diet group, $p \leq 0.05$ ($n = 9$)

No differences were detected among diet groups for any of the drug treatments.

WK-C: control group, ($n = 9$); WK-B: blueberry group, ($n = 9$); Ach: acetylcholine; MFA: mefenamic acid; L-NMMA: L-N^G-monomethyl arginine

Figure 4.2. Effect of Diet on the Fmax* in the Absence or Presence of Inhibitors in the WK Rats



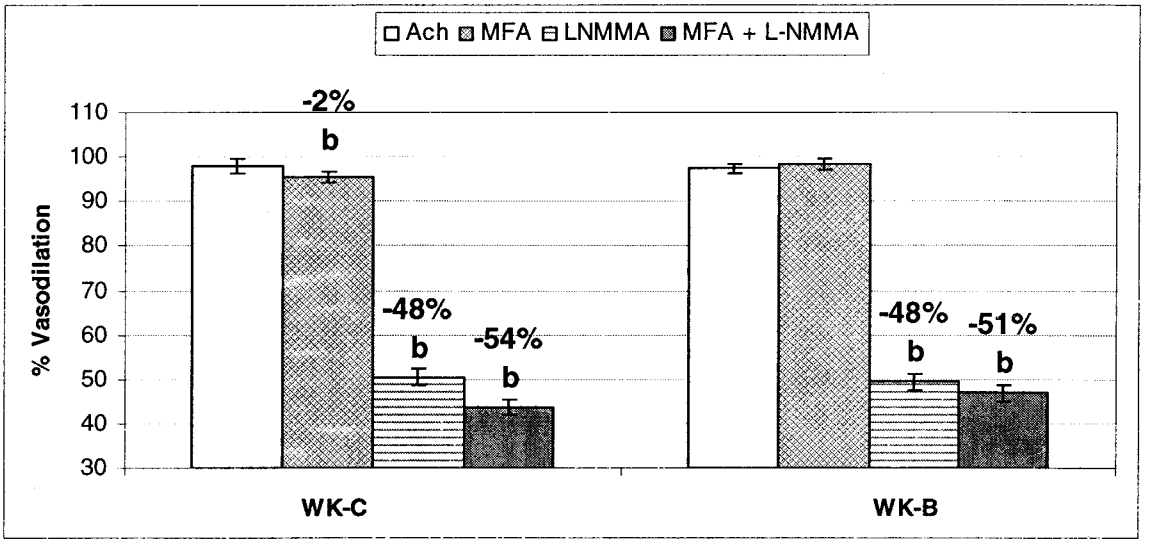
* Mean ± SEM
WK-C: control group, (n = 9); WK-B: blueberry group, (n = 9); Ach: acetylcholine; MFA: mefenamic acid; L-NMMA: L-N^G-monomethyl arginine
No differences were detected among diet groups for any of the drug treatments.

4.1.3. Effect of Inhibitors on Maximum Vasodilation Force (Fmax)

Figure 4.3 presents the changes in the Fmax due to the addition of inhibitors within the same diet group. The changes were calculated by subtracting the Fmax in the presence of the inhibitors from the Fmax in the absence of inhibitors. Inhibition of the COX pathway with MFA elicited a statistically significant reduction of maximum vasodilation by 2% in the WK-C group, ($p \leq 0.05$). In the WK-B group, the maximum vasodilation was increased by 1% in the presence of MFA, but this increase was not statistically significant, ($p = 0.46$). Inhibition of NOS with L-NMMA reduced maximum vasodilation by 48% in both diet groups. The difference in Fmax elicited by L-NMMA was statistically significant compared with the Fmax in the absence of the inhibitor, ($p \leq 0.05$).

The presence of both inhibitors, MFA and L-NMMA, triggered a statistically significant reduction in maximum vasodilation force by 54% in the WK-C group and 51% in the WK-B group, $p \leq 0.05$.

Figure 4.3. Fmax* in the Absence or Presence of Inhibitors in the WK Rats

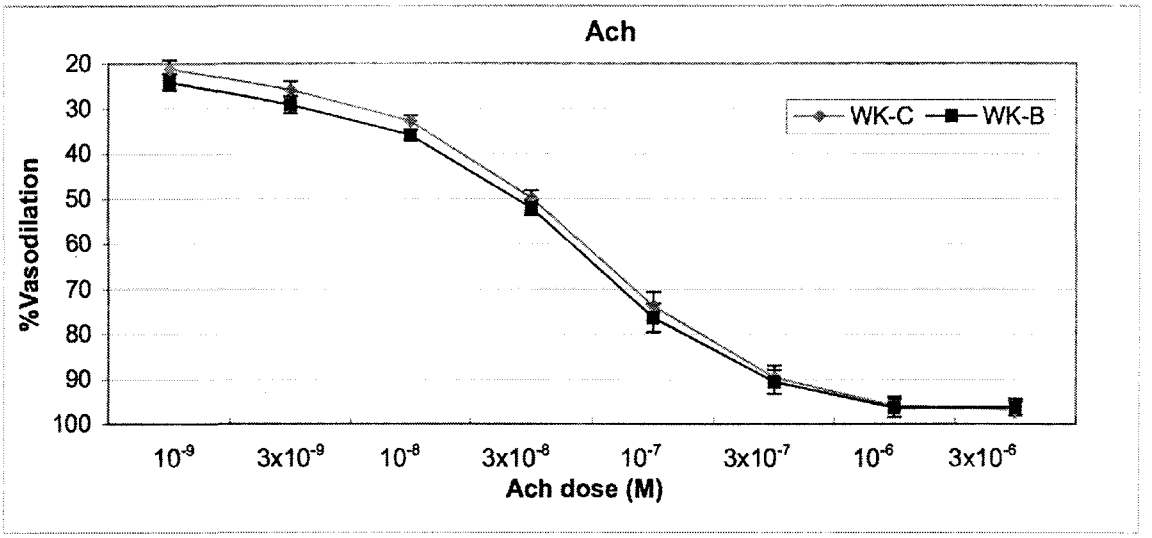


* Mean ± SEM
[b] Statistically significant compared to Ach treatment, $p \leq 0.05$ (n = 9)
WK-C: control group, (n = 9); WK-B: blueberry group, (n = 9); Ach: acetylcholine; MFA: mefenamic acid; L-NMMA: L-N^G-monomethyl arginine

4.1.4. Effect of Diet on Vasodilation Force, Ach Dose-Response Curves

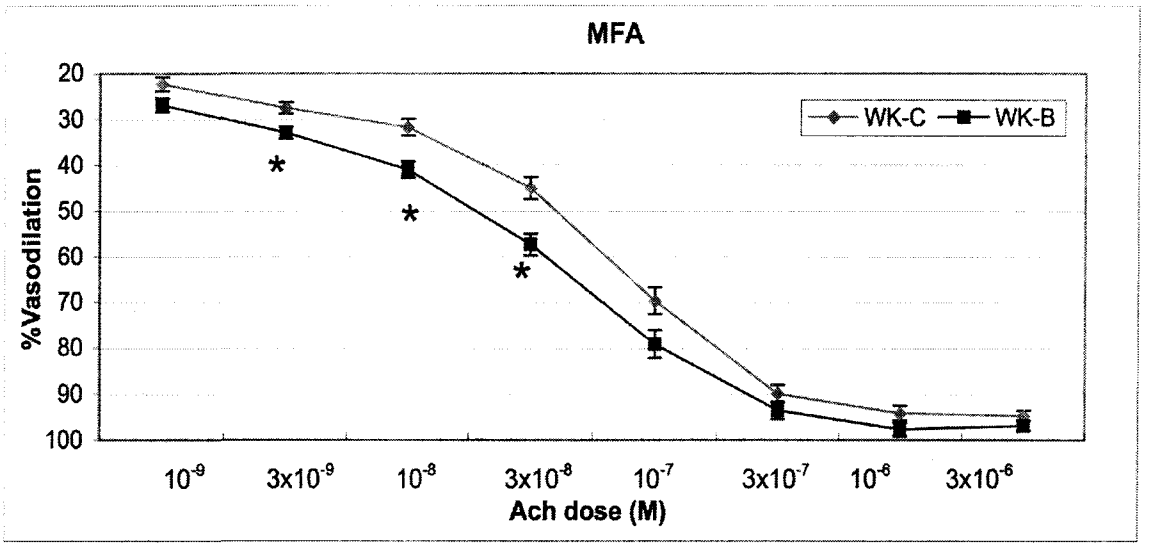
Figures 4.4, 4.5, 4.6 and 4.7 display the dose-response curves of the Ach-induced vasodilation in the aortic rings in the absence or presence of MFA, L-NMMA or both MFA and L-NMMA. In the absence of inhibitors the vasodilation force was not significantly different between the two diet groups (Figure 4.4). In the presence of COX inhibitor MFA, the aortic rings of the WK-B group tended to develop higher vasodilation than the WK-C group throughout the dose-response curve, which was significant only for the 2nd, 3rd and 4th Ach doses (3×10^{-9}, 10^{-8} and 3×10^{-8}M), ($p \leq 0.05$) (Figure 4.5).

Figure 4.4. Ach Dose-Response Curve in the Absence of Inhibitors in the WK Rat Aorta



WK-C: control group, (n = 9); WK-B: blueberry group (n = 9); Ach: acetylcholine

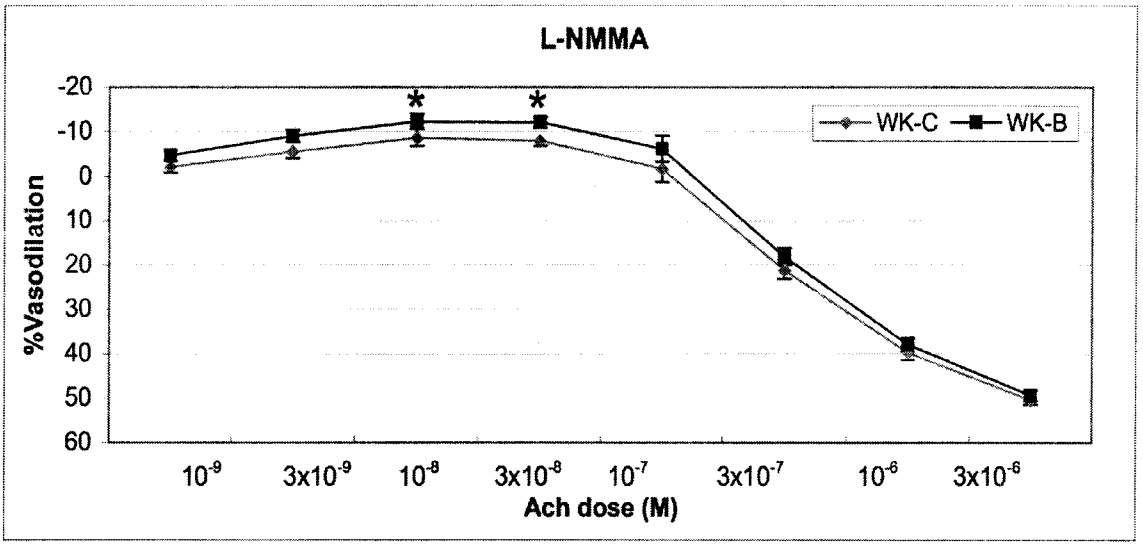
Figure 4.5. Ach Dose-Response Curve in the Presence of MFA in the WK Rat Aorta



* Statistically significant compared to WK-C group, p≤0.05
WK-C: control group, (n = 9); WK-B: blueberry group, (n = 9); Ach: acetylcholine; MFA: mefenamic acid

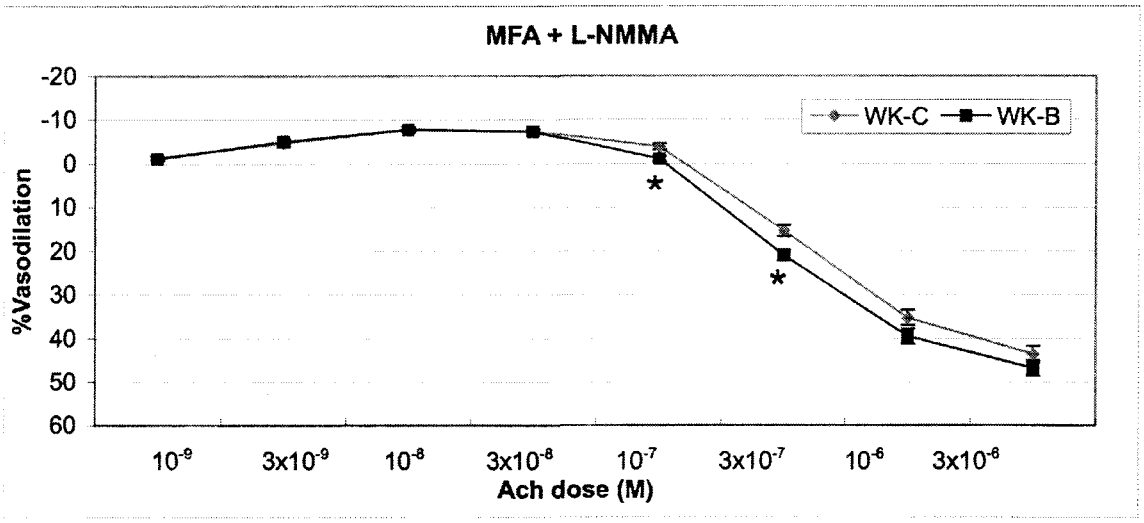
In the presence of NOS inhibitor L-NMMA, the aortic rings from the WK-B group tended to develop lower vasodilation force throughout the dose-response curve, which was significantly lower for the 3rd and 4th Ach dose (10^{-8} and 3×10^{-8}M), ($p\leq0.05$) (Figure 4.6). When both inhibitors were added, the vasodilation force was almost identical between the two diet groups for the first four Ach doses, but the WK-B group exhibited significantly higher vasodilation force at the 5th and 6th Ach dose (10^{-7} and 3×10^{-7}M), ($p\leq0.05$) (Figure 4.7).

Figure 4.6. Ach Dose-Response Curve in the Presence of L-NMMA in the WK Rat Aorta



* Statistically significant compared to WK-C group, $p\leq0.05$
WK-C: control group, (n = 9); WK-B: blueberry group, (n = 9); Ach: acetylcholine; L-NMMA: L-N^G-monomethyl arginine

Figure 4.7. Ach Dose-Response Curve in the Presence of both MFA and L-NMMA in the WK Rat Aorta



* Statistically significant compared to WK-C group, p≤0.05
WK-C: control group, (n = 9); WK-B: blueberry group, (n = 9); Ach: acetylcholine; MFA: mefenamic acid; L-NMMA: L-N^G-monomethyl arginine

4.1.5. Effect of Diet on Vessel Sensitivity (pD_2)

The vessel sensitivity expressed as pD_2 values for the aortic rings of WK rats fed control and wild blueberry-enriched diet is displayed in Table 4.2 and Figure 4.8. The pD_2 in the absence of inhibitors was significantly lower in the WK-B group (7.41 ± 0.02) than the WK-C group (7.49 ± 0.02), ($p \leq 0.05$). The pD_2 in the WK-B group (7.51 ± 0.03) in the presence of MFA was not different than the pD_2 of the WK-C group (7.56 ± 0.03), ($p = 0.21$). The addition of L-NMMA did not elicit any difference among diet groups, WK-B (6.81 ± 0.10) and WK-C (6.65 ± 0.10), ($p = 0.28$). Similarly, the pD_2 in the presence of both MFA and L-NMMA was not different among diet groups, WK-B group (6.65 ± 0.13) vs. WK-C group (6.56 ± 0.13), ($p = 0.63$).

Table 4.2. Effect of Diet on the pD_2* of WK Rat Aorta in the Absence or Presence of Inhibitors

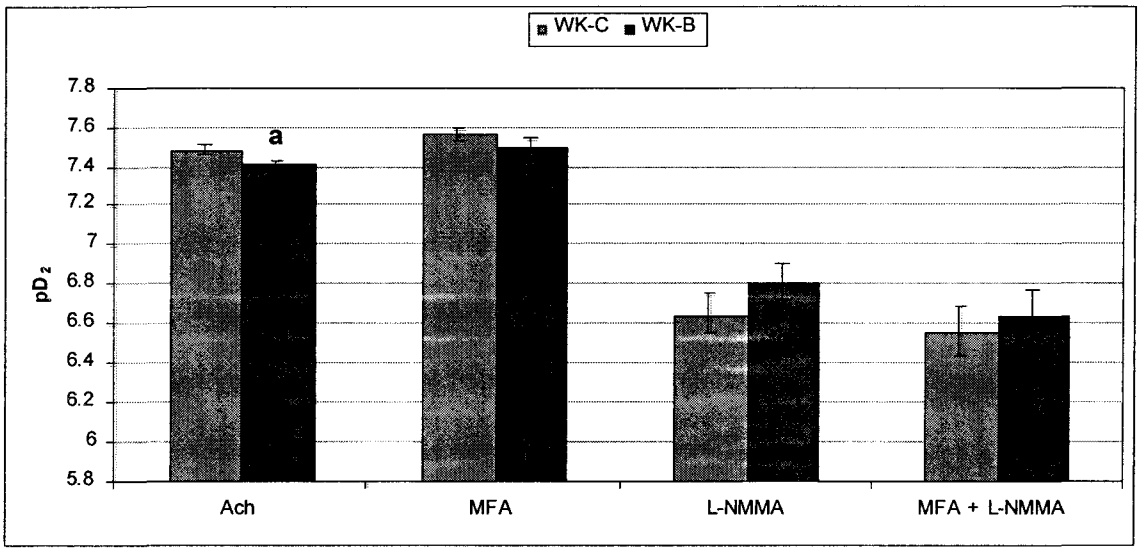
Diet group	Ach	MFA	L-NMMA	MFA + L-NMMA
WK-C	7.49 ± 0.02	7.56 ± 0.03	6.65 ± 0.10	6.56 ± 0.13
WK-B	7.41 ± 0.02[a]	7.51 ± 0.03	6.81 ± 0.10	6.65 ± 0.13

* Mean ± SEM

[a] Statistically significant compared to WK-C, $p \leq 0.05$

WK-C: control group, (n = 9); WK-B: blueberry group, (n = 9); Ach: acetylcholine; MFA: mefenamic acid; L-NMMA: L-N^G-monomethyl arginine

Figure 4.8. Effect of Diet on the pD_2* of WK Rat Aorta in the Absence or Presence of Inhibitors



* Mean ± SEM

[a] Statistically significant compared to WK-C, $p \leq 0.05$

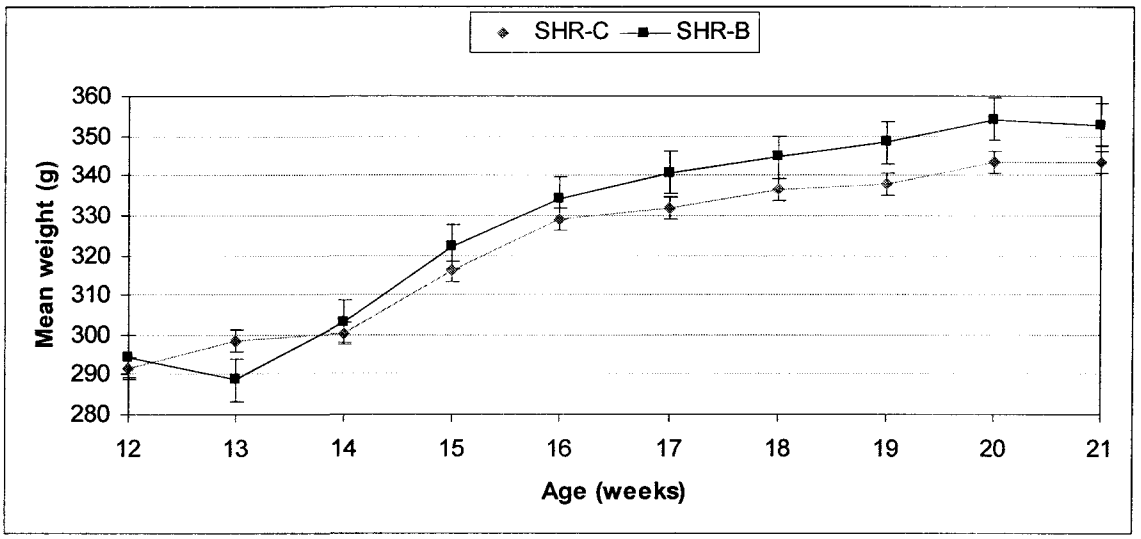
WK-C: control group, (n = 9); WK-B: blueberry group (n = 9); Ach: acetylcholine; MFA: mefenamic acid; L-NMMA: L-N^G-monomethyl arginine

4.2. Spontaneously Hypertensive Rats

4.2.1. Rat Growth and Weight

Figure 4.9 represents the growth rate of SHRs fed control (SHR-C) and wild blueberry-enriched (SHR-B) diet from 12 to 21 weeks of age. The rate of growth during the nine week time period was not significantly different between the two diet groups. The final mean body weights at the end of the diet study were 343 ± 2.70 g and 353 ± 5.34 g for the SHR-C and SHR-B group respectively, which were not statistically significant, ($p = 0.13$). Also, no statistically significant difference was found for food intake of the two diet groups, 20 ± 0.36 g in the SHR-C group and 20 ± 0.36 g in the SHR-B group, ($p = 0.45$) .

Figure 4.9. Growth Rate of SHRs (Weekly Weight*)



* Mean ± SEM (g)
SHR-C: control group, (n = 10); SHR-B: blueberry group, (n = 10)

4.2.2. Effect of Diet on Maximum Vasodilation Force (Fmax)

The effect of diet on the Fmax in response to Ach a) in the absence of inhibitors or in the presence of b) the COX-pathway inhibitor mefenamic acid (MFA) or c) the NO-pathway inhibitor L-N^G-monomethyl arginine (L-NMMA) or d) both inhibitors added simultaneously, is presented in Table 4.3 and Figure 4.10. The Fmax in response to Ach observed in the SHR-B group (92.13 ± 0.56) was significantly lower than the Fmax observed in the SHR-C group (94.63 ± 0.56), ($p \leq 0.05$), while the Fmax observed for the SHR-B group (102.17 ± 0.57) in the presence of MFA was significantly higher than in the SHR-C group ((97.76 ± 0.57), ($p \leq 0.05$). In presence of L-NMMA, Fmax, did not differ significantly among diet groups (SHR-B group: 46.45 ± 0.49 vs. SHR-C group: 45.36 ± 0.49), ($p = 0.16$). Finally, when both inhibitors were present, Fmax was similar in the two diet groups (SHR-B group: 53.60 ± 0.89 vs. SHR-C group: 53.49 ± 0.89), ($p = 0.93$).

Table 4.3. Effect of Diet on the Fmax* in the Absence or Presence of Inhibitors in the SHRs

Diet group	Fmax Ach	Fmax Ach MFA	Fmax Ach L-NMMA	Fmax Ach MFA + L-NMMA
SHR-C	94.63 ± 0.56	97.76 ± 0.57[b]	45.39 ± 0.49[b]	53.49 ± 0.89[b]
SHR-B	92.13 ± 0.56[a]	102.17 ± 0.57[a,b]	46.45 ± 0.49[b]	53.60 ± 0.89[b]

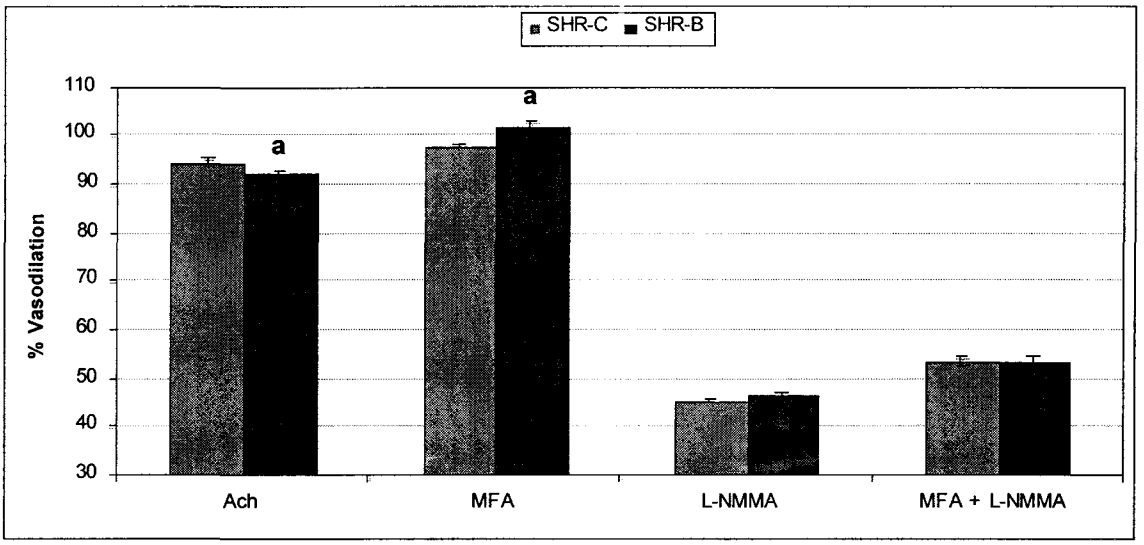
* Mean ± SEM

[a] Statistically significant compared to SHR-C group, $p \leq 0.05$

[b] Statistically significant compared to Ach without the presence of inhibitors within the same diet group, $p \leq 0.05$ (n = 10)

SHR-C: control group, (n = 10); SHR-B: blueberry group, (n = 10); Ach: acetylcholine; MFA: mefenamic acid; L-NMMA: L-N^G-monomethyl arginine

Figure 4.10. Effect of Diet on the Fmax* in the Absence or Presence of Inhibitors in the SHRs



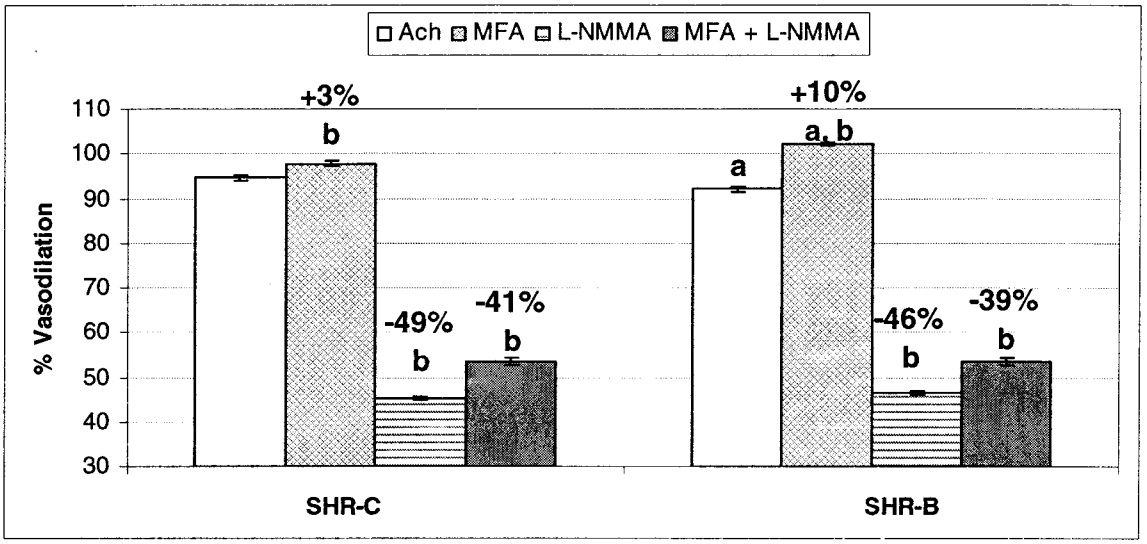
* Mean ± SEM
[a] Statistically significant compared to SHR-C, p≤0.05
SHR-C: control group, (n = 10); SHR-B: blueberry group, (n = 10); Ach: acetylcholine; MFA: mefenamic acid; L-NMMA: L-N^G-monomethyl arginine

4.2.3. Effect of Inhibitors on Maximum Vasodilation Force (Fmax)

Figure 4.11 presents the changes in the Fmax due to the addition of inhibitors within the same diet group. The changes were calculated by subtracting the Fmax in the presence of the inhibitors from the Fmax in the absence of inhibitors. Inhibition of the COX pathway with MFA resulted in a significant increase of maximum vasodilation by 3% in the SHR-C group, while in the SHR-B group, the presence of MFA triggered a 10% increase in the Fmax, (p≤0.05). Inhibition of the NOS with L-NMMA reduced maximum vasodilation by 49% in the SHR-C group and by 46% in the SHR-B group. The change in Fmax induced by L-NMMA was statistically significant in both diet groups, (p≤0.05). The presence of both inhibitors, MFA and L-NMMA, caused a significant reduction in maximum

vasodilation force by 41% in the SHR-C group and 39% in the SHR-B group, ($p \leq 0.05$).

Figure 4.11. Fmax* in the Absence or Presence of Inhibitors in the SHRs

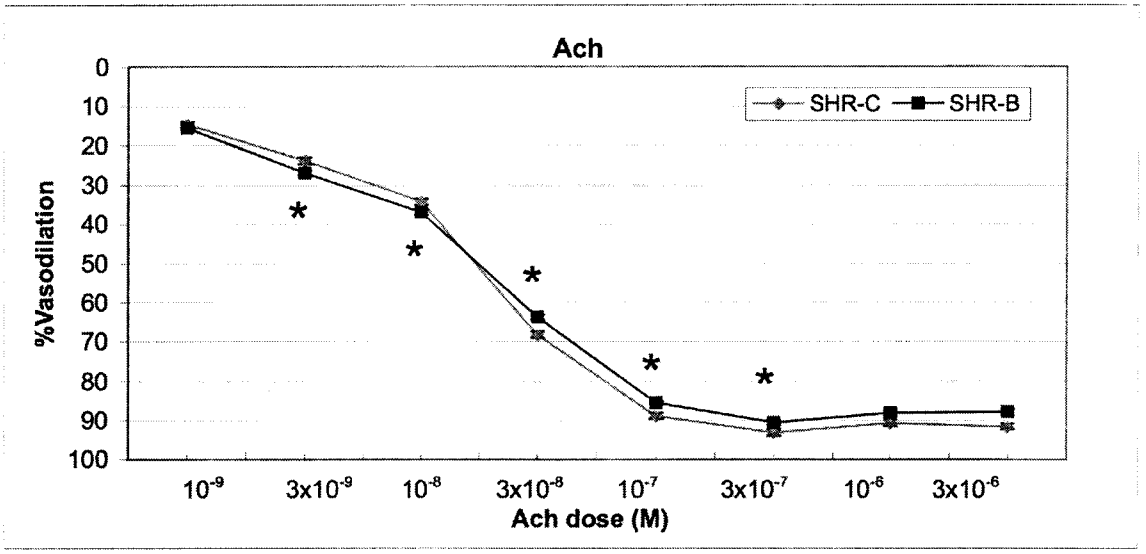


* Mean ± SEM
[a] Statistically significant compared to SHR-C, $p \leq 0.05$
[b] Statistically significant compared to Ach treatment, $p \leq 0.05$ (n = 10)
SHR-C: control group, (n = 10); SHR-B: blueberry group, (n = 10); Ach: acetylcholine; MFA: mefenamic acid; L-NMMA: L-N^G-monomethyl arginine

4.2.4. Effect of Diet on Vasodilation Force, Ach Dose-Response Curves

Figures 4.12, 4.13, 4.14 and 4.15 display the dose-response curves of the Ach-induced vasodilation in the aortic rings in the absence or presence of MFA, L-NMMA or both MFA and L-NMMA. In the absence of inhibitors, the vasodilation force was significantly greater in the SHR-B group for the 2nd and 3rd Ach dose (3×10^{-9} and 10^{-8}M), but significantly lower for the 4th, 5th and 6th Ach dose (10^{-7}, 3×10^{-7} and 3×10^{-6}M), ($p \leq 0.05$) (Figure 4.12).

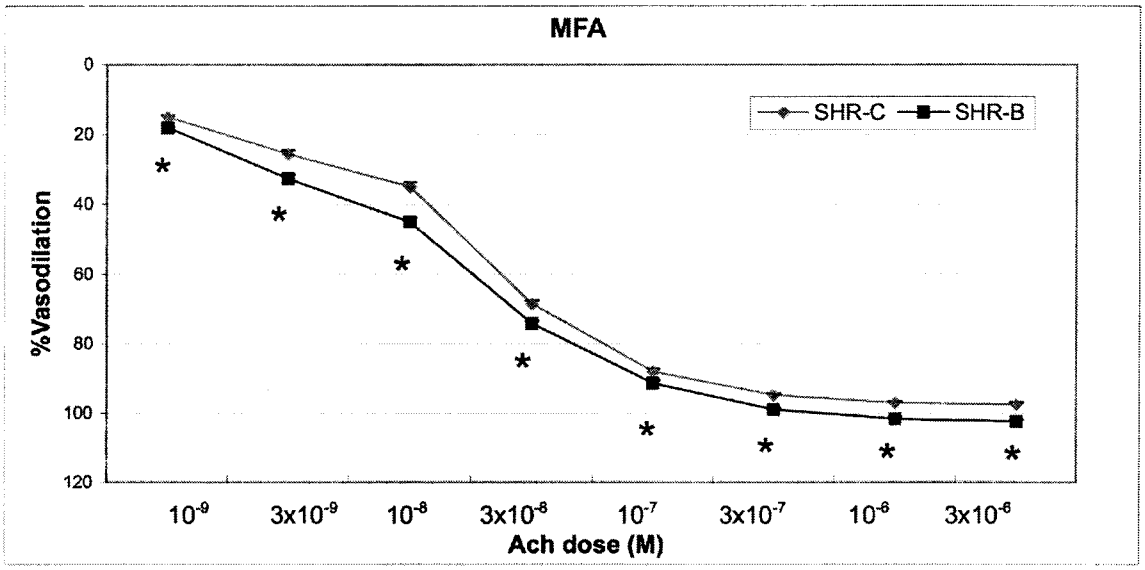
Figure 4.12. Ach Dose-Response Curve in the Absence of Inhibitors in the SHR Aorta



* Statistically significant compared to SHR-C, p≤0.05
SHR-C: control group, (n = 10); SHR-B: blueberry group, (n = 10); Ach: acetylcholine

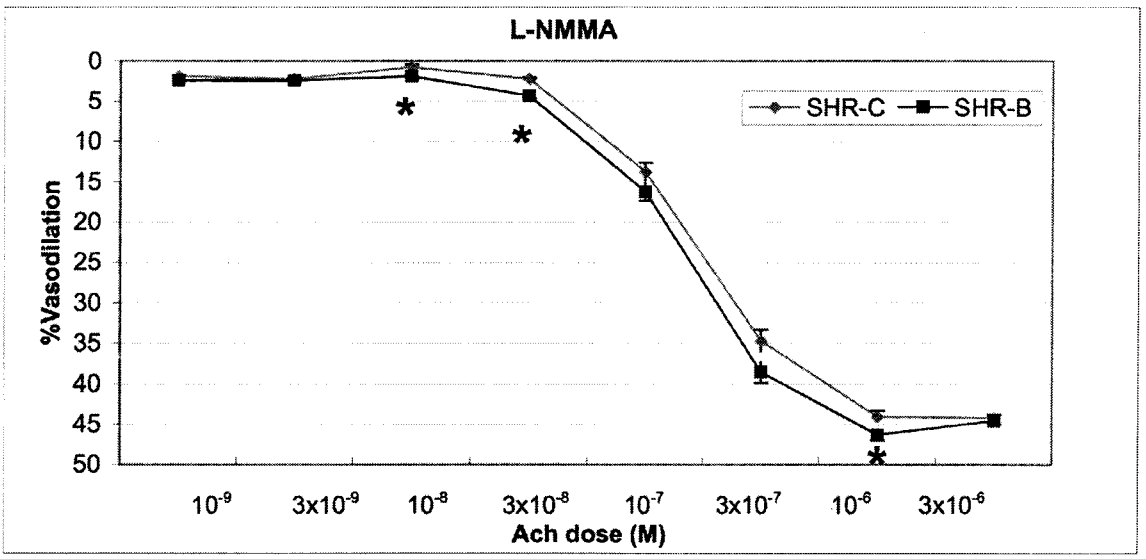
In the presence of COX inhibitor MFA, the aortic rings from the SHR-B developed significantly greater vasodilation for all Ach doses (p≤0.05) (Figure 4.13), while in the presence of NOS inhibitor L-NMMA, the aortic rings from the SHR-B group developed significantly greater vasodilation at the 3rd, 4th and 7th Ach doses only (10^{-8}, 3×10^{-8} and 10^{-6}M), (p≤0.05) (Figure 4.14). When both inhibitors were added, the SHR-B aortic rings exhibited significantly higher vasodilation response than the SHR-C rings, at the first four Ach doses (10^{-9}, 3×10^{-9}, 10^{-8} and 3×10^{-8}M), (p≤0.05) (Figure 4.15).

Figure 4.13. Ach Dose-Response Curve in the Presence of MFA in the SHR Aorta



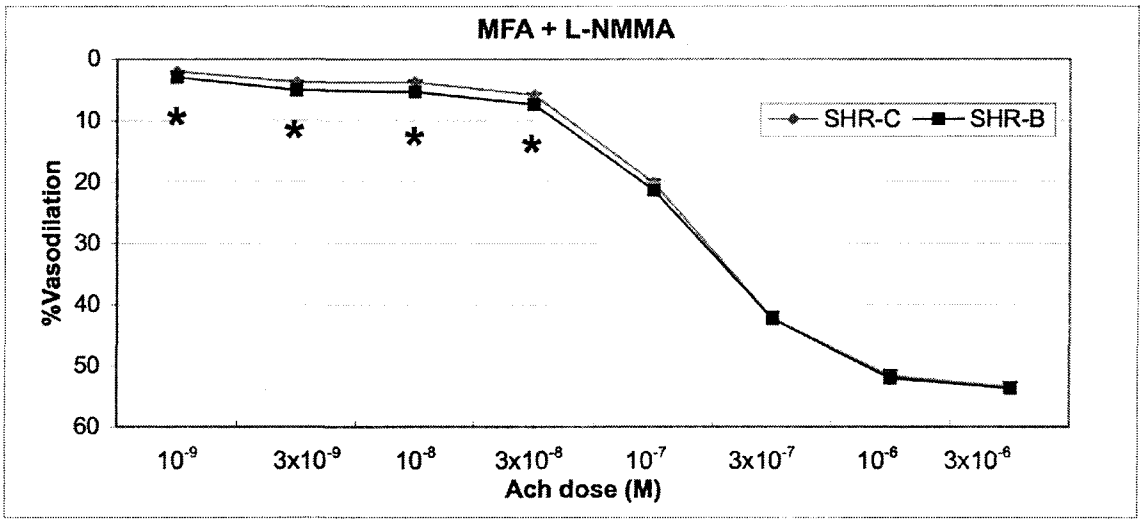
* Statistically significant compared to SHR-C group, p≤0.05
SHR-C: control group, (n = 10); SHR-B: blueberry group, (n = 10); Ach: acetylcholine; MFA: mefenamic acid

Figure 4.14. Ach Dose-Response Curve in the Presence of L-NMMA in the SHR Aorta



* Statistically significant compared to SHR-C group, p≤0.05
SHR-C: control group, (n = 10); SHR-B: blueberry group, (n = 10); Ach: acetylcholine; L-NMMA: L-N^G-monomethyl arginine

Figure 4.15. Ach Dose-Response Curve in the Presence of both MFA and L-NMMA in SHR Aorta



* Statistically significant compared to control group, p≤0.05
SHR-C: control group, (n = 10); SHR-B: blueberry group, (n = 10); Ach: acetylcholine; MFA: mefenamic acid; L-NMMA: L-N^G-monomethyl arginine

4.2.5. Effect of Diet on Vessel Sensitivity (pD_2)

The vessel sensitivity expressed as pD_2 values for the aortic rings of SHRs fed control and wild blueberry-enriched diet is displayed in Table 4.4 and Figure 4.16. The pD_2 in the absence of inhibitors did not differ among diet groups (SHR-B group: 7.54 ± 0.02 vs. SHR-C group: 7.59 ± 0.02), (p = 0.11). In the presence of MFA the pD_2 in the SHR-B group (7.72 ± 0.02) was significantly greater than the SHR-C group (7.63 ± 0.02), (p≤0.05). The pD_2 was also found to differ significantly among diet groups in the presence of L-NMMA, SHR-B: 7.17 ± 0.02 vs. SHR-C: 7.04 ± 0.02, (p≤0.05). Finally, the pD_2 in the presence of both MFA and L-NMMA was not different between diet groups (SHR-B: 7.20 ± 0.04 and SHR-C: 7.25 ± 0.04), (p = 0.38).

Table 4.4. Effect of Diet on the pD_2* of SHR Aorta in the Absence or Presence of Inhibitors

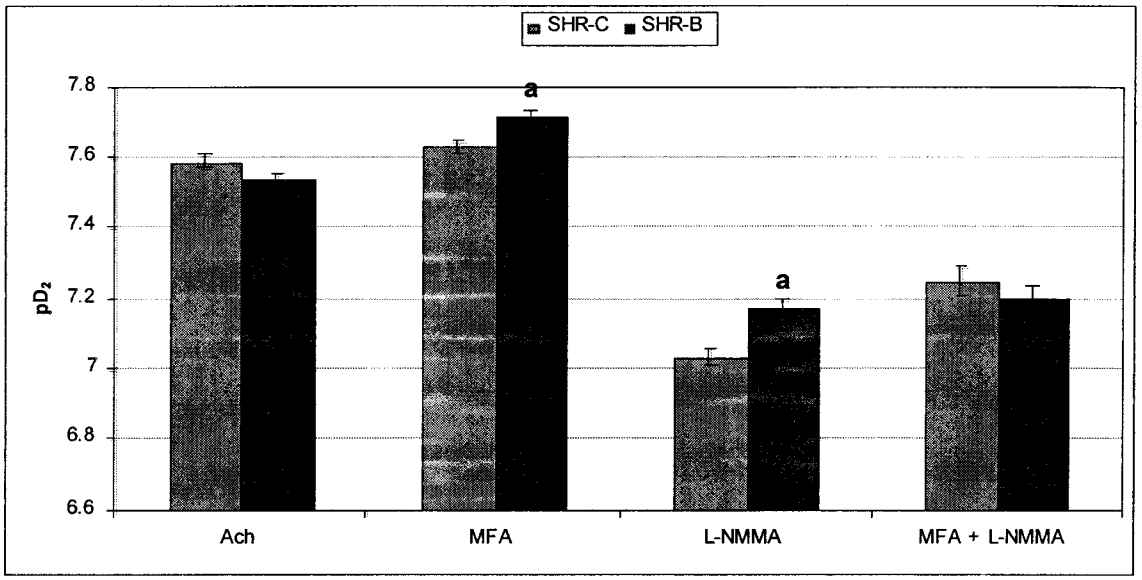
Diet group	Ach	MFA	LNMMA	MFA + L-NMMA
SHR-C	7.59 ± 0.02	7.63 ± 0.02	7.04 ± 0.02	7.25 ± 0.04
SHR-B	7.54 ± 0.02	7.72 ± 0.02[a]	7.17 ± 0.02[a]	7.20 ± 0.04

* Mean ± SEM

[a] Statistically significant compared to SHR-C, p≤0.05

SHR-C: control group, (n = 10); SHR-B: blueberry group, (n = 10); Ach: acetylcholine; MFA: mefenamic acid; L-NMMA: L-N^G-monomethyl arginine

Figure 4.16. Effect of Diet on the pD_2* of SHR Aorta in the Absence or Presence of Inhibitors



* Mean ± SEM

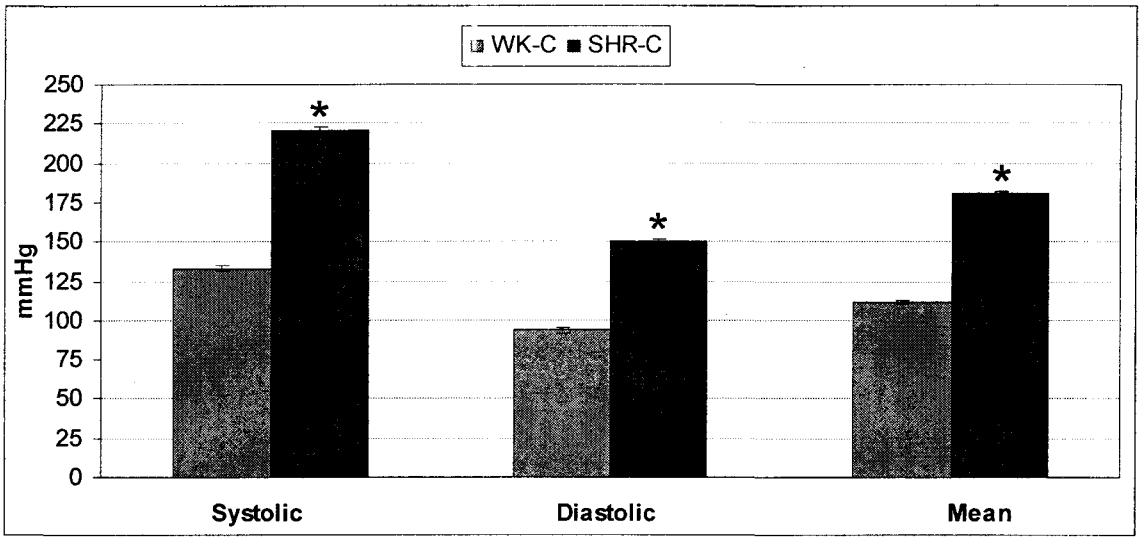
[a] Statistically significant compared to SHR-C, p≤0.05

SHR-C: control group, (n = 10); SHR-B: blueberry group, (n = 10); Ach: acetylcholine; MFA: mefenamic acid; L-NMMA: L-N^G-monomethyl arginine

4.3. Blood pressure (BP)

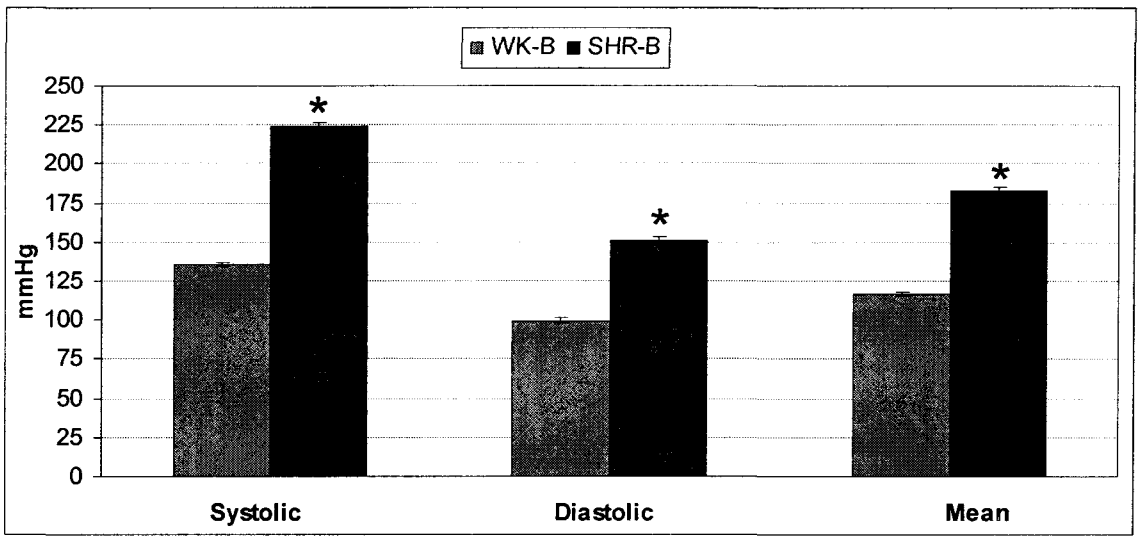
Blood pressure was measured in WK rats and SHRs fed a control or wild blueberry-enriched diet for nine weeks. Measurements of systolic, diastolic and mean arterial BP were taken. Even though systolic, diastolic and mean BP was significantly different between rat strains ($p \leq 0.05$), no significant difference was detected between dietary treatments (Figure 4.17, 4.18). Table 4.5 presents the mean systolic, diastolic and mean arterial BP in the WK rats. The mean systolic BP in the WK-B group (136 ± 1.55 mmHg) was not significantly different from the WK-C group (133 ± 1.55 mmHg), ($p = 0.29$). Similarly, the mean diastolic BP in the WK-B group (100 ± 1.95 mmHg) did not differ significantly from the WK-C group (94 ± 1.95 mmHg), ($p = 0.52$). Finally, no significant difference was observed in the mean arterial BP in the WK-B group (117 ± 1.78 mmHg) vs. the WK-C group (112 ± 1.78 mmHg), ($p = 0.08$). Table 4.6 presents the mean systolic, diastolic and mean arterial BP in the SHRs. No significant differences were detected between diet groups in the mean systolic BP (SHR-B group: 224 ± 3.66 mmHg vs. SHR-C group: 221 ± 3.66 mmHg, $p = 0.56$), the mean diastolic BP (SHR-B group: 151 ± 1.43 mmHg vs. SHR-C group: 150 ± 1.43 mmHg, $p = 0.52$) and the mean arterial BP (SHR-B group: 183 ± 2.10 mmHg vs. SHR-C group: 180 ± 2.09 mmHg, $p = 0.47$).

Figure 4.17. Systolic, Diastolic and Mean Arterial BP[1] in WK-C and SHR-C



[1]Mean ± SEM (mmHg)
* Statistically significant compared to WK-C, $p \leq 0.05$
WK-C: control group (n = 10); SHR-C: control group (n = 10)

Figure 4.18. Systolic, Diastolic and Mean Arterial BP[1] in WK-B and SHR-B



[1]Mean ± SEM (mmHg)
* Statistically significant compared to WK-B, $p \leq 0.05$
WK-B: blueberry group (n = 10); SHR-B: blueberry group (n = 10)

Table 4. 5. Effect of Diet on the Systolic, Diastolic and Mean Arterial BP[1] in the WK Rats

Diet group	Systolic BP	Diastolic BP	Mean BP
WK-C	133 ± 1.55	94 ± 1.95	112 ± 1.77
WK-B	136 ± 1.55	100 ± 1.95	117 ± 1.77

[1] Mean ± SEM (mmHg)
WK-C: control group, (n = 10); WK-B: blueberry group, (n = 10); BP: blood pressure
No significant differences were detected among WK-C and WK-B.

Table 4.6. Effect of Diet on the Systolic, Diastolic and Mean Arterial BP[1] in the SHRs

Diet group	Systolic BP	Diastolic BP	Mean BP
SHR-C	221 ± 3.66	150 ± 1.43	180 ± 2.09
SHR-B	224 ± 3.66	151 ± 1.43	183 ± 2.09

[1] Mean ± SEM (mmHg)
SHR-C: control group, (n=10); SHR-B: blueberry group (n = 10); BP: blood pressure
No significant differences were detected among SHR-C and SHR-B.

CHAPTER 5

DISCUSSION

5.1. Summary of Results

The aim of the present study was to evaluate the *ex vivo* effect of nine week dietary treatment with wild blueberries (*Vaccinum angustifolium*) on the arterial functional properties of the hypertensive and normotensive young adult rat. Wild blueberries were provided at 8% (w/w) of total diet content, which is equivalent to daily human consumption of a half cup of fresh-wild blueberries (Norton *et al.*, 2005). To our knowledge there are no feeding studies on the effect of wild blueberries on vasomotion besides the studies conducted in this laboratory (Kalea *et al.*, 2005; Norton *et al.*, 2005; Clark, 2007). Previous studies conducted in our laboratory reported that wild blueberries can reduce vasoconstriction induced by phenylephrine (Phe) in the aorta of young normotensive young Sprague-Dawley (SD) rats, an action that requires an intact and functional endothelium. In SD rats of this age, wild blueberries do not seem to exert their effect on vasoconstriction in response to Phe through cell membrane receptor-agonist interactions (Norton *et al.*, 2005). Additionally, wild blueberries were shown to affect the endothelium dependent-vasodilation via the NO pathway in young normotensive SD and by modulating the production and/or the activity of COX-derived products in the young hypertensive SHR aorta (Clark, 2007). The same study confirmed that in the normotensive SD rat, the endothelium-dependent vasodilation is primarily mediated by NO. Additionally, wild

blueberries exert their effect on acetylcholine (Ach)-induced vasodilation by modulating cell membrane receptor-agonist interactions (Clark, 2007).

The objectives of the current study were to examine the possible effect of wild blueberries on endothelium-dependent vasodilation in aortas with dysfunctional endothelium from young adult SHRs with full-blown hypertension, and their controls, the normotensive WK rats with functional endothelium, in an attempt to dissect the biochemical mechanism that wild blueberries may employ on vasodilation in the young adult hypertensive rat and normotensive rat.

We documented that in the young adult normotensive WK, wild blueberries do not affect maximum endothelium-dependent vasodilation in response to Ach. However, wild blueberries seem to modulate the cell membrane receptor-agonist interactions in response to Ach in the above strain. In the young adult SHR, wild blueberries reduce the maximum vasodilation force in response to Ach, an effect which is mediated by the COX pathway, as shown by the increased maximum vasodilation force in response to Ach with the COX-pathway inhibition. Furthermore, the SHR-B aortic rings exhibited greater Ach-induced vasodilation at lower Ach doses ($3x10^{-9}$ and 10^{-8}M) compared to SHR-C. Additionally, wild blueberries seem to have an effect on the receptor-agonist interactions in response to Ach in the young adult SHR when either NO or COX pathway is inhibited. Finally, wild blueberries did not have a significant effect on the systolic, diastolic and mean arterial blood pressure (BP) in either strain of rats. Hence, the effect of wild blueberries on vasodilation may be strain-dependent and/ or depend on the physiological state of the aorta (functional vs. dysfunctional

endothelium) as shown by the differential effect upon the functional endothelium of WK and dysfunctional endothelium of SHR. Additionally, the effect of wild blueberries on vasodilation does not seem to be associated with regulation of BP in the young adult SHR and WK rat.

5.2. Wistar Kyoto Rats

5.2.1. Effect of Wild blueberries on Endothelium-Dependent Vasodilation in Response to Ach

In the present study, the Ach-induced vasodilation in the aortas between diet groups was similar in the young adult WK rats. In agreement with these findings a recent study conducted in our laboratory on young SD rats did not reveal any significant effect of wild blueberry-enriched diet on Ach-induced vasodilation (Clark, 2007). It can be therefore suggested that wild blueberries do not seem to influence the muscarinic receptor in smooth muscle cells. However, wild blueberry diets affect the arterial biomechanical properties by suppressing the α_1-adrenergic receptor-agonist vasoconstriction induced by Phe, as documented by an earlier dietary study on young normotensive SD rats conducted in our laboratory (Norton *et al.*, 2005). Thus, wild blueberries have differential effects on different receptors of vascular cells.

The involvement of NO and COX pathways in endothelium-mediated vasodilation in response to Ach was evaluated by their inhibition with L-NMMA and MFA respectively. Additionally, both inhibitors were used simultaneously to examine the potential role of NO-COX interaction on vasodilation. The presence of any of

the inhibitors, either separately or simultaneously, did not have any significant effect on the vasodilation between the two diet groups. These findings suggest that wild blueberries do not affect to a great extend the NO or COX-mediated pathways in the young adult normotensive WK when provided at 8% of total diet composition and for the period of nine weeks. The inhibitory effect of L-NMMA on vasodilation was more pronounced in young SD rats fed wild blueberries versus SD rats fed a control diet (Clark, 2007), implying that wild blueberries show potential in enhancing or preserving NO bioavailability in animals with functional endothelium. However, in the present study, utilizing a different strain of normotensive rat, the WK, did not seem to affect the vasodilation pathways studied.

It is not clear why wild blueberries seem to be involved in the NO pathway in the SD but not in the WK rats, since both strains have a functional endothelium. Interspecies variability in the type, density and conformation of agonist receptors, as well as in the structure of the vascular wall itself, may explain the above observations. Additionally, the age difference, i.e. young SD vs. young adult WK, may also contribute to the above phenomenon. A study by Vizioli *et al.* (2005), indicated that vasodilation of the thoracic aorta of 12 to 14 week-old WK rats in response to Ach, besides NO, involves COX-derived prostanoids as indicated by inhibition with indomethacin (Vizioli *et al.*, 2005). Another study by Heymes *et al.* (2000), documented that in old WK rats (24 vs. 4 months of age) vasoconstrictor prostanoids may reduce Ach-induced vasodilation (Heymes *et al.*, 2000). Hence,

it is possible that in the WK rats the action of prostanoids may shift from vasodilatory in younger age to vasoconstrictor in older aged rats.

Rapoport and Williams (1996) proposed prostacyclin (PGI_2) as the endothelium-derived contracting factor responsible for Ach induced contractions in 7 to12 months-old WK rats (Rapoport and Williams, 1996). Moreover, in the aorta of WK rat older than 15 weeks, PGI_2 receptor (IP) agonist cannot evoke IP receptor-mediated relaxations (Levy, 1980; Rapoport and Williams, 1996).

In the present study, the maximum vasodilation was not different between diet groups after COX pathway inhibition in the WK rat. Similarly, wild blueberries did not have a significant effect on maximum vasodilation under the COX inhibition in the SD rat (Clark, 2007). The inhibition of the COX pathway significantly reduced vasodilation in the WK-C group, possibly by preventing the generation of COX-derived vasoconstrictors. However, COX-inhibition did not alter significantly the maximum vasodilation in the WK-B group, suggesting that wild blueberries may cancel out the vasoconstrictor effect of COX through an unknown as yet mechanism.

The inhibition of NO pathway did not elicit a different response in the maximum vasodilation force between diet groups in the young adult WK rat. However, the possibility that wild blueberries may protect NO bioavailability in the WK rat, cannot be excluded in the view of previous findings that wild blueberry exhibited the potential to preserve NO in the SD rat (Clark, 2007).

The vasorelaxant and the NO-protective effect of isolated flavonoid compounds in normotensive animal models with functional endothelium, has been

demonstrated by several studies (Andriambeloson *et al.*, 1998; Zenebe *et al.*, 2003; Benito *et al.*, 2002; Chung *et al.*, 1993; Nakamura *et al.*, 2002). Delphinidin was shown to elicit an endothelium-dependent vasodilatory effect in WK rat aorta, which was completely mediated by NO activity (Andriambeloson *et al.*, 1998). Additionally, incubation of WK femoral artery with red wine polyphenol powder, particularly rich in proanthocyanidins, increased NO bioactivity and vasorelaxation (Zenebe *et al.*, 2003). Quercetin and structurally related compounds showed vasorelaxant activity in the thoracic WK rat aorta by suppressing the Ca^{2+}- and Phe-induced contractions (Chung *et al.*, 1993). In SD rats, black currant concentrate, rich in delphinidin and cyanidin (Nakamura *et al.*, 2002) as well as a diet rich in dealcoholated red wine, quercetin or catechin (Benito *et al.*, 2002), elicited a vasodilatory effect mediated by the NO pathway. Similarly, incubation of SD rat aortic rings with the flavonoid-rich artichoke leaf organic subfraction was shown to enhance the NO mediated vasodilator response to Ach (Li *et al.*, 2004).

Our observations from the inhibition of the NO and COX pathway are in agreement with the work of Vizioli *et al.* (2005), which showed that in the thoracic aorta of 12 to 14 week-old WK, the Ach-induced vasodilation was inhibited by L-NMMA treatment and was partially reduced by the non-selective COX-inhibitor indomethacin. Additionally, treatment of rings with both L-NMMA and indomethacin also reduced the vasodilation response to Ach (Vizioli *et al.*, 2005). The only difference is that in our study, the inhibition of the COX-pathway in the WK fed wild blueberry, did not significantly alter the Ach-induce vasodilation

force. Vassale *et al.* (2003) indicated that a pronounced release of NO is expected when COX is inhibited in order to compensate for the reduced PGI_2 levels, whereas the vasodilator prostaglandins seem to have no capacity to modulate NO release in the endothelial cells when NO synthase is inhibited (Vassale *et al.*, 2003). This finding further supports the role of wild blueberries on preserving NO, since wild blueberry treatment may potentiate a greater NO bioavailability, in comparison to the control. Fundamentally, in both human subjects and animal models with functional endothelium, NO is the primary mediator of the Ach-induced vasodilation (Taddei and Salvetti, 2002). Our study confirmed that inhibition of NO synthase by L-NMMA leads to a far greater attenuation of vasodilation force than the inhibition of COX.

Overall, although the wild blueberry treatment did not elicit any significant change in the vasodilation force in the normotensive WK rat aorta, the possibility that it may act on preserving the NO bioavailability in the WK rat, cannot be excluded.

5.2.2. Vessel Reactivity

Vessel reactivity or sensitivity, pD_2, is an index of cell membrane-receptor agonist interactions. Vessel reactivity is the negative log of the concentration of the agonist required to inhibit 50% of the vessel response, EC_{50}. In our study, pD_2 is used as an indication of whether wild blueberries can affect the cell membrane-receptor agonist interactions. In the WK rat, Ach induced a significant difference on the pD_2 values among the two diet groups. The WK-B group showed a decrease in the vessel sensitivity as compared to the control. This

finding suggests a possible interaction between membrane receptors and the muscarinic agonist Ach in the WK-B group. Our observations on vessel sensitivity on the WK rat agree with Clark (2007), who also reported an effect of wild blueberries on the vessel sensitivity of the SD rats (Clark, 2007).

Previous work in Dr. Klimis-Zacas' laboratory reported that blueberries can modulate the structure of the extracellular matrix (EC) of young male SD rat aortas by increasing the concentration of glycosaminoglycans (GAGs) and decreasing the sulfation of all GAG-type molecules (Kalea *et al.*, 2005). Glycosaminoglycans are structural components of the glycocalyx, which coats the luminal surface of the vascular endothelial cells. The glycocalyx is considered a first line of protection against atherogenic damage of the endothelium (van den Berg *et al.*, 2006). Glycosaminoglycans participate in the structural organization of the EC and in the regulation of several vascular functions. A possible role in signal transduction pathways has been attributed to the effect of GAG sulfation, in Ach-receptor clustering and therefore sensitivity (Mc. Donnell and Grow, 2000). Furthermore, several enzymes such as eNOS, superoxide dismutase (SOD) or angiotensin converting enzyme (ACE), growth factors and chemokines, all with a principal role in plasma and vessel wall homeostasis are present in the vascular environment. Therefore the glycocalyx is a vital player of endothelial function and homeostasis (van den Berg *et al.*, 2006). Based on the findings that wild blueberries were shown to alter the structure of the EC, a possible effect of blueberries on endothelial and vascular smooth muscle signal transduction pathways in the WK rat can be implied.

5.3. Spontaneously Hypertensive Rats

5.3.1. Effect of Wild Blueberries on Endothelium-Dependent Vasodilation in Response to Ach

This study was aimed at clarifying the possible effect of wild blueberries on endothelium-dependent vasodilation in the aorta of young adult SHR with a dysfunctional endothelium. The SHRs develop hypertension without exception between the age of 7 to 15 weeks (Yamori, 1984). The present study on arterial functional properties was conducted on 21 week-old SHRs after 9 weeks of dietary treatment in order to investigate the role of wild blueberries in fully-developed hypertension.

Acetylcholine dose-response curves in the absence and in the presence of NO and COX inhibitors were constructed to study the effect of wild blueberries on vasodilation pathways in the SHR aorta. In the absence of inhibitors, the dose-response curve revealed that the vasodilation force was higher in the SHR-B group at the lower Ach doses but lower at the higher Ach doses (Figure 4.12). Overall, a significantly lower vasodilation force in response to Ach in the SHR-B group versus the SHR-C group was observed. Hence, wild blueberries act to increase the vasodilatory response to Ach at lower doses, whereas this effect is reversed at the higher Ach doses. In the SHR, higher Ach doses than those required for vasodilation, can induce vasoconstriction due to EDCF release, but also due to a direct effect on VSMC (Boulanger *et al.*, 1994; Luscher and Vanhoutte, 1986). However, in the young SHR wild blueberries did not alter the endothelium-dependent vasodilation in response to Ach in the absence of

inhibitors. The same study indicated that the vasodilation force developed in the young SHR fed wild blueberry was higher at the Ach doses 10^{-8} and 3×10^{-8}M but not different at any other Ach dose (Clark, 2007). Hence, the vasodilatory effect of wild blueberries at lower Ach doses in young and young adult SHR is similar. However, at higher Ach doses, the wild blueberry diet seems to have a different and probably age-dependent effect on vasodilation.

In SHRs younger than 14 weeks old, the endothelium-mediated vasorelaxation seems to be similar to normotensive rats of the same age and therefore young SHRs may be considered as normotensive before full development of hypertension (Cappelli-Bigazzi *et al.*, 1997). Acetylcholine-mediated vascular relaxation, even after treatment with the COX inhibitor indomethacin, did not differ significantly among normotensive and young SHR, while the release of COX-dependent vasoconstrictors occurred only in vessels of aged normotensive or SHR animals and in response to higher concentrations of Ach (10^{-5} and 10^{-6}M) (Koga *et al.*, 1989). Hence, it is possible that a greater release of COX-derived vasoconstrictors occurs at higher Ach doses in the young adult SHR vs. the young SHR, which can mask any possible effect of wild blueberries on vasodilation. Cyclooxygenase-derived vasoconstrictors generated in response to higher Ach doses may be responsible for the observed decline in the wild blueberry effect on vasodilation occurring in higher Ach doses ($3x10^{-8}$ to $3x10^{-6}$M). It has been reported that in the 12 to 14 week-old SHR, endoperoxides (PGH_2) account for the reduced endothelium dependent contraction, whereas in the 72 week-old rat impaired NO formation and/ or increased NO inactivation

seem to be involved as well (Kung and Luscher, 1995). Furthermore, in one year old SHR, PGI_2 induced by Ach was shown to act as a contracting and not a relaxing factor (Gluais *et al.*, 2005), probably due to a decreased response of the IP receptor. In the aorta of older than 15 weeks WK or SHR, IP receptor agonists cannot evoke relaxations (Levy, 1980; Rapoport and Williams, 1996). The expression of the IP receptor gene decreases with age in the SHR and WK rat as well (Numaguchi *et al.*, 1999). Hence the aging process, by favoring a vasoconstrictor state in the aorta, may contribute to the decreased vasodilation observed in the young adult SHR fed wild blueberry, possibly due to the interaction of wild blueberry with one or more factors that induce age-dependent changes such as prostaglandins or their receptors. Furthermore, it can be suggested that the greater vasodilation observed at the lower Ach doses in the SHR may reflect a potential effect of blueberries on initial precontraction rather than an effect on vasodilation. As observed by Norton *et al.* (2005), wild blueberries were shown to suppress the α_1-adrenergic receptor-agonist vasoconstriction induced by Phe in the SD rat (Norton *et al*, 2005). This possibility cannot be ignored, even though the effect of wild blueberries may be different among strains. Studies on the effect of wild blueberries on the vasoconstriction in the SHR are not currently available to clarify this possibility.

The inhibition of the COX pathway produced higher vasodilation in the SHR-B aorta versus the SHR-C, suggesting a possible effect of wild blueberries on the COX-derived vasodilatory prostanoids in the young adult SHR. On the other hand, the inhibition of NO synthesis with L-NMMA did not induce any difference

in the vasodilation among the diet groups, implying that wild blueberries do not affect NO bioavailability in the young adult SHR aorta. Wild blueberries have been shown to preserve NO bioavailability in the young normotensive SD rats (Clark, 2007). However, the endothelial dysfunction in the SHR seems to limit the wild blueberry potential in preserving NO bioavailability. In the SHR the endothelium-dependent vasodilation seems to be impaired due to a reduced availability of NO (Kerr *et al.*, 1999; Xiao and Pang, 1994). Besides the reduced NO release, the increased release of EDCF(s) also accounts for the blunted endothelium-dependent relaxations to Ach in the SHR aorta (Luscher and Vanhoutte, 1986).

In our study, when the release of the COX-derived vasodilators such as PGI_2 and vasoconstrictors such as TXA_2, was inhibited by MFA, wild blueberries exhibited a vasorelaxant effect. An enhanced release of NO may occur with the inhibition of PGI_2 generation (Vassale *et al.*, 2003). Wild blueberries may further aid this compensatory mechanism by preserving NO bioavailability due to their antioxidant properties (Mazza *et al.*, 2002; Kay and Holub, 2002). In the present study, when vascular rings were treated with MFA, the vasodilation force was increased in both diet groups, confirming the finding by Taddei *et al.* (1998) that COX inhibition can increase or normalize the blunted Ach-induced vasodilation of dysfunctional endothelium (Taddei *et al.*, 1998). When both NO and COX pathways were inhibited, vasodilation was decreased in both diet groups, but there was no difference in the vasodilatory response between them. The NO and COX pathways constantly interact and therefore manipulation of one pathway

may have an effect on both (Mollace *et al.*, 2005). As previously mentioned, in our study, when both NO and COX pathways were operating, the vasodilation force was decreased in the SHR-B group, but it cannot be determined whether this effect is due to a direct influence of wild bueberries on COX derived vasoconstrictors, or to an indirect adverse effect of COX-derived vasoconstrictors on NO bioavailability.

Cyclooxygenase is overexpressed in the SHR, resulting in an increased production of vasoconstrictor prostanoids (Vanhoutte *et al.*, 2005), as well as an increased ROS levels (Katusic, 1996). The excessive production of superoxide anion results in the increased oxidative stress in the vascular walls of SHR, which has been postulated to mediate endothelial dysfunction (Kerr *et al.*, 1999; Yang *et al.*, 2002; Cuzocrea *et al.*, 2004). Antioxidant dietary treatment has been shown to improve the endothelial function in the SHR (Maccha and Mustafa, 2005). The endothelium-dependent vasodilator response to Ach and overall endothelial function can be restored in SHR treated with γ-tocotrienol (Newaz *et al.*, 2003) or in hypertensive humans treated with vitamin C (Taddei *et al.*, 1998), as a result of the antioxidant and free radical scavenging activity of the vitamins. Besides antioxidant vitamins, several flavonoid compounds have demonstrated a protective activity against oxidative stress. The antioxidant activity of cyanidin-3 galactoside and several quercetin glycosides isolated from cranberry was comparable to vitamin E (Yan *et al.*, 2002). By scavenging peroxynitrite ($ONOO^-$) cyanidin-3-O-glucoside from blackberry juice had a protective *in vitro* effect against endothelial dysfunction and vascular failure (Serraino *et al.*, 2003). Red

wine polyphenols, delphinidin and cyanidin can directly scavenge ROS and prevent the ROS platelet derived growth factor$_{AB}$ (PDGF$_{AB}$)-induced formation in cultured vascular smooth muscle cell (VSMC). Additionally malvidin and peonidin although they did not scavenge ROS, prevented their cellular formation (Oak *et al.*, 2006).

Wild blueberries have been endowed with antioxidant properties (Mazza *et al.*, 2002; Kay and Holub, 2002); therefore they may protect endothelium function through ROS scavenging. Besides free radical scavenging, wild blueberry components can modulate other mechanisms towards a vasoprotective result. The inhibition of protein kinase C and cAMP release, inhibition of cyclic nucleotide phosphodiesterase or decreased Ca^{2+} may also contribute to the vasodilatory effect of flavonoids in a manner related to the flavonoid structure (Duarte *et al.*, 1993). Wine, grape juice, grape skin extracts and several plant extracts were shown to produce endothelium-dependent relaxation *in vitro* mediated by the NO and through an increase in cGMP levels (Fitzpatrick *et al.*, 1993; Fitzptrick *et al.*, 1995). Quercetin was shown to reduce the α_1-adrenergic receptor mediated contractile response (Ajay *et al.*, 2006) and to enhance eNOS activity, besides decreasing NADPH oxidase mediated superoxide generation (Sanchez *et al.*, 2006). Cyanidin-3-glucoside was shown to increase eNOS expression and NO release in bovine vascular endothelial cells (Xu *et al.*, 2004a) and to regulate phosphorylation of eNOS and the protein kinase Akt (Xu *et al.*, 2004b). Delphinidin and cyanidin suppressed *in vitro* lipopolysacharide-induced COX-2 expression in murine macrophages (Hou *et al.*, 2005). Cyanidin, cyanidin

3-galactoside and cyanidin 3-glucoside from *Amelanchier* fruits were shown to inhibit *in vitro* COX-1 and -2 in a dose-dependent manner (Adhikari *et al.*, 2005). Therefore, a wild blueberry-enriched diet potentially has a positive effect on endothelium-dependent vasodilation in the young adult SHR, via promoting NO-mediated vasodilation in an antioxidant-dependent or -independent manner. However, the beneficial effect of wild blueberries on Ach-induced vasodilation in the young adult SHR seems to be masked by the high activity of COX-derived vasoconstrictor factors on this genetic model of endothelial dysfunction. A longer dietary treatment and/or a higher concentration of wild blueberries in the diet may be necessary to elicit a beneficial effect. Furthermore, in the SHR model, a strong genetic predisposition underlies endothelial dysfunction and therefore, a protective role of wild blueberries on vasodilation may be observed in a preventive, rather than a therapeutic dietary treatment.

5.3.2. Vessel Reactivity

No significant differences in vessel sensitivity among diet groups were observed in the young adult SHR in the absence of the inhibitors or in the presence of both inhibitors. However, when either L-NMMA or MFA were added separately, the vessel sensitivity in the blueberry group was higher than in the control diet group. These findings strengthen further the hypothesis that, wild blueberries act on the agonist receptor interactions possibly through modification of EC at the GAG level, as previously reported (Kalea *et al.*, 2005). Components of the EC matrix are protected by antioxidant activity, as EC SOD has been shown to protect

heparin/heparan sulfate (Kliment *et al.*, 2008) and hyaluronan (Gao *et al.*, 2008) from oxidative fragmentation. These studies provide an explanatory link between the antioxidant effect of wild blueberries and EC protection. It is not clear, however, why wild blueberries exhibit this effect only when NO and COX pathways are inhibited separately, but not simultaneously.

In comparison with age matched WK rats, the SHR aortic tissue has an increased total GAG concentration as well as different GAG composition and sulfation pattern, changes that have been implicated in the development of hypertension (Risler *et al.*, 2003). Provided the potential of wild blueberries to alter the GAG profile in the normotensive rat to a less atherogenic one (Kalea *et al.*, 2005), it is possible that wild blueberries show a similar potential in the SHR.

5.4. Blood Pressure

To our knowledge this is the first attempt to study the effect of wild blueberries on the arterial blood pressure (BP) of the young adult normotensive WK rat and SHR. Blood pressure was directly measured in WK rats and SHR, fed a control or blueberry-enriched diet (8% w/w) for nine weeks. Measurements of systolic, diastolic and mean arterial BP were taken. No significant difference between the diet groups was found in either strain of rats. Our study reveals that at the level of 8% of diet for nine weeks, wild blueberries do not have any significant effect on the BP of young adult WK and SHR. The amount of 8% wild blueberries in the animal diet is equivalent to half a cup of blueberries or 120 g of human daily consumption. Several studies (Mizutani *et al.*, 1999; Shindo *et al.*, 2007; Duarte

et al., 2001; Sanchez *et al.*, 2006; Sakaida *et al.*, 2007; Liu *et al.*, 2003) have revealed the hypotensive effect of isolated flavonoid components, also found in wild blueberries, but in our study we proposed to document the effect of the wild blueberries on BP and therefore we utilized the whole fruit.

Red wine polyphenols induced a decrease in the BP of 12 week old WK rats, after a short term oral administration (Diebolt *et al.*, 2001). Extract of wine phenolics were shown to reduce BP elevation in Stroke Prone Spontaneously Hypertensive Rat (SHRSP) after an eight-week treatment with phenolic-enriched diet (Mizutani *et al.*, 1999). Anthocyanins from purple corn, sweet potato and red radish administered at the level of 1% of diet for 15 weeks starting at the age of 5 weeks were shown to reduce BP in SHR (Shindo *et al.*, 2007). Quercetin administration for five weeks reduced BP and enhanced the endothelium-dependent vasodilation in response to Ach by reducing oxidative stress in the 17 week-old SHR, but not in the WK rat (Duarte *et al.*, 2001). In addition to lowering BP and heart rate of SHR, and enhancing endothelium-dependent vasodilation, quercetin increased eNOS activity and decreased NADPH oxidase-mediated superoxide generation (Sanchez *et al.*, 2006). Blood pressure of SHR was reduced after treatment with blueberry leaf extract, which contained 18.7% tannins (Sakaida *et al.*, 2007). Various tannins were shown to reduce BP in SHR, via non-specific inhibition of angiotensin converting enzyme (ACE) (Liu *et al.*, 2003). In addition, polyphenolic compounds, such as flavan-3-ols and procyanidins, isolated from cocoa were found to reduce significantly ACE activity

in vitro due to competition for enzyme-active sites with synthetic substrates rather than a direct antioxidant effect on ACE (Actis-Gorretta *et al.*, 2003).

Similarly to animal studies, the observations from human studies as related to BP reduction, vary depending upon the bioactive compounds, their source, as well as the administration dose, method and duration (McAnulty *et al.*, 2005; Grassi *et al.*, 2005; Aviram *et al.*, 2001; Aviram *et al.*, 2004). Daily consumption of 250 g of blueberries for three weeks did not elicit any change in the BP of chronic smokers, although an alleviation of oxidative stress was observed (McAnulty *et al.*, 2005). A 15-day daily consumption of 100 g flavanol-rich dark chocolate (88mg flavonols/100g of dark chocolate) decreased BP and improved endothelium-dependent relaxation in patients with essential hypertension (Grassi *et al.*, 2005). In hypertensive patients, daily consumption for a two week period of 50 ml pomegranate juice, containing 1.5 mmol of total polyphenols, resulted in a 36% decrease in serum ACE and a 5% reduction in systolic BP (Aviram *et al.*, 2001). A reduction in BP was also observed after long term (3 years) consumption of pomegranate juice by patients with carotid artery stenosis (Aviram *et al.*, 2004). The beneficial effect of pomegranate juice in BP and ACE is attributed to their antioxidant properties.

The oxidative stress in SHR can be reduced with antihypertensive treatment (Lazaro *et al.*, 2005). On the other hand, the reduction of antioxidant stress can regulate high BP. Seven-month dietary treatment of young weanling SHR with an antioxidant diet rich in vitamins E and C, Zn and Se, reduced BP and renal interstitial inflammation (Rodriguez-Iturbe *et al.*, 2003). Additionally, antioxidant

drug treatment for three weeks was found to regulate BP and improve eNOS and iNOS in vascular, renal and cardiac tissues of SHR, but not of WK rat, indicating the contribution of oxidative stress in experimental hypertension and the compensatory upregulation of eNOS and iNOS in SHR (Vaziri *et al.*, 2000). In 20 to 22 week-old SHR a three-month dietary treatment with γ-tocotrienol resulted in increased NO activity and reduced BP (Newaz *et al.*, 2003). The antihypertensive effect of γ-tocotrienol in the above study was attributed to its antioxidant-radical scavenging properties.

Beyond any improvement in the oxidative stress, the restoration of endothelial dysfunction does not necessarily lead to normalization of BP. The thromboxane receptor (TP) agonist ifetroban, was shown to normalize Ach-induced relaxations in the 16-week old SHR, however, it did not have any effect on blood pressure (Tesfamariam and Ogletree, 1995). A 12-week treatment with 1 or 2 mg/kg/day simvastatin, a cholesterol biosynthesis inhibitor, administered orally improved Ach-induced endothelium-dependent vasodilation, but did not elicit any significant effect on the systolic BP in the 20 week-old SHR (de Sotomayor *et al.*, 1999). Similarly, an 8–week treatment with 200 mg/kg/day L-carnitine and propionyl-L-carnitine improved carbachol-induced relaxation, without preventing the development of hypertension of the 12-week old SHR (Bueno *et al.*, 2005). Additionally, resveratrol dosage mimicking moderate red wine consumption, was shown to improve endothelium-dependent relaxations to Ach in SHR after a 4-week administration due to improved NO bioavailability, but did not affect systolic BP in the SHR (Rush *et al.*, 2007). These studies indicate that the improvement

in endothelial-dependent vasodilation does not necessarily ensure a reduction in BP in the SHR.

Endothelial dysfunction is not specific to essential hypertension, but instead commonly observed in connection with the major cardiovascular risk factors (Vita *et al.*, 1990; Taddei and Salvetti, 2002). Dissociation between the degree of endothelial dysfunction and arterial BP values was indicated for human hypertension (John and Schmieder, 2000). Moreover no correlation was detected between BP values and endothelium-dependent vasodilation (Panza *et al.*, 1993). Finally, BP reduction per se is not associated with improvement of endothelium-dependent vasodilation (Panza *et al.*, 1993; Taddei and Salvetti, 2002). Hence, it is plausible that wild blueberries, despite their potential in affecting endothelial function, do not have the ability to reduce BP in the SHR and WK rats, at least after an 8% w/w wild blueberry enriched diet for 9 weeks. Wild blueberries are unable to compensate for the reported impairment of the endothelium of the SHR, which is characterized by an imbalance between vasoconstrictors and vasodilators (Mombuli and Vanhoutte, 1999), an increased superoxide production (Kerr *et al.*, 1999) and other ROS, as well as COX-1 derived endoperoxides (Yang *et al.*, 2003). Furthermore, taking into consideration that bioactive compounds, also found in blueberries have shown a positive effect on BP when studied individually or in high concentrations, the possibility cannot be excluded that a competition of bioactive compounds in the complex matrix of the whole wild blueberry fruit may account for the lack of any effect on BP. A diet enriched with higher amounts of wild blueberries fed for

longer time may be necessary to document a significant effect on blood pressure of SHR. This hypothesis needs to be examined with future studies.

5.5. Limitations and Future Recommendations

In the present study, we investigated the effect of a wild blueberry-enriched diet on major vasodilation pathways and arterial blood pressure in the young adult (21 week old) SHR and WK rat. The SHR is a model of endothelial dysfunction and hypertension, widely used in other organ system experimental settings, but presenting multiple metabolic defects. Their vasculature is structurally altered due to genetically predetermined impairments, increased release and activity of vasoconstrictors, as well as increased oxidative stress.

The dietary effect of wild blueberries was observed on the COX pathway of the SHR, resulting in enhanced vasodilation and implying that wild blueberries exhibit the potential to maintain arterial functional properties in this strain. Their positive effect(s) are limited by the multiple impairments of the SHR vascular system. Thus, these positive effects cannot extend to the level of BP either, at least at the level of 8% of diet for a nine week period. Hence, higher wild blueberry content in the diet or longer treatment periods can be suggested for future work. On the other hand, studies on the effect of wild blueberries on the vasoconstrictor pathways, would be useful in providing a complete picture of the interactions of wild blueberry treatment with the vascular tone and the balance of vasodilatory and vasoconstrictor substances on endothelial functions. In the same vein, biochemical measurements of major vasodilators, NO and prostacyclin, as well

vasoconstrictors, TXA_2 and endoperoxides, can offer more insight into the mechanism(s) that wild blueberries employ to affect vascular tone.

5.6. Significance

Considering the magnitude of the health and financial consequences of CVD, scientific search for more effective and at the same time more affordable means of tackling CVD has become more than a necessity. The endothelium, being the primary site of dysfunction in all types of CVD, appears to be the ideal target for a preventive approach. Diet is among the lifestyle modifications, along with exercise and smoking cessation, which present the potential to maintain and/ or restore endothelial function. Various dietary bioactive compounds have attracted an increasing research interest over the last decade due to their acclaimed health benefits. Among these compounds, flavonoids in particular, have been positively associated with cardiovascular health protection. Accumulating evidence has documented a positive link between consumption of foods containing bioactive compounds, also described as functional foods, and their effect on health beyond nutrition. Wild blueberries rank high among functional foods due to their high antioxidant capacity, as well as the plethora of bioactive compounds they contain, which may have multiple effects on health beyond antioxidant protection.

Our laboratory has been the first to investigate the potential role of a wild blueberry-enriched diet on the arterial functional properties *ex vivo* and specifically vasodilation, as well as the arterial blood pressure of the young adult

SHR and its normotensive control, the WK rat. Results of the present study demonstrate that wild blueberries can enhance vasodilation in the SHR via a COX-pathway shift towards a reduced vasoconstrictor or increased vasodilator profile. Wild blueberries also produced a differential activity between the two strains, implying that their role in vasodilation depends on the state of the endothelium, functional vs. dysfunctional. These observations are of significant practical importance for pathological conditions with endothelial dysfunction and may have further implications for the prevention and treatment of CVD.

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APPENDIX

Arterial Functional Property Experiments

PROTOCOL: Two Ach dose-response curves

Preload application (1,5 g for 3mm arterial ring)
Preconditioning: Ach 17 µl 10^{-5}M (10^{-8}M in the tissue bath)
Phe 17 µl 10^{-5}M (10^{-8}M in the tissue bath)
Inhibitors: MFA 17 µl 10^{-2}M (10^{-5} in the tissue bath)

		MFA 17 µl 10^{-2}M	**MFA** 17 µl 10^{-2}M

- *10 min*
- Wash out 4-5x.
- **Inhibitors:** MFA 17 µl 10^{-2}M (10^{-5} in the tissue bath)

		MFA 17 µl 10^{-2}M	**MFA** 17 µl 10^{-2}M

- *25 min*
- Readjust to 0 g.
- *5 min*

Precontraction: Phe 17 µl 10^{-3}M (10^{-6}M in the tissue bath)
~10 min

1st Ach curve
1. Ach 17 µl 10^{-6}M (10^{-9}M in the tissue bath) –*6 min*
2. Ach 51 µl 10^{-6}M ($3x10^{-9}$M in the tissue bath) –*6 min*
3. Ach 17 µl 10^{-5}M (10^{-8}M in the tissue bath) –*6 min*
4. Ach 51 µl 10^{-5}M ($3x10^{-8}$M in the tissue bath) –*6 min*
5. Ach 17 µl 10^{-4}M (10^{-7}M in the tissue bath) –*6 min*
6. Ach 51 µl 10^{-4}M ($3x10^{-7}$M in the tissue bath) –*6 min*
7. Ach 17 µl 10^{-3}M (10^{-6}M in the tissue bath) –*6 min*
8. Ach 51 µl 10^{-3}M ($3x10^{-6}$M in the tissue bath) –*6 min*

- Wash out 4-5x.
- **Inhibitors: L-NMMA** 17 µl 10^{-1}M (10^{-4} in the tissue bath)
MFA 17 µl 10^{-2}M (10^{-5} in the tissue bath)

L-NMMA 17 µl 10^{-1}M	**L-NMMA** 17 µl 10^{-1}M	**L-NMMA** 17 µl 10^{-1}M **MFA** 17 µl 10^{-2}M	**L-NMMA** 17 µl 10^{-1}M **MFA** 17 µl 10^{-2}M

- *25 min*
- Readjust to 0 g.
- *5 min*

Precontraction: Phe 17 µl 10^{-3}M (10^{-6}M in the tissue bath)
~10 min

2nd Ach curve

1. Ach 17 µl 10^{-6}M (10^{-9}M in the tissue bath) –*6 min*
2. Ach 51 µl 10^{-6}M ($3x10^{-9}$M in the tissue bath) –*6 min*
3. Ach 17 µl 10^{-5}M (10^{-8}M in the tissue bath) –*6 min*
4. Ach 51 µl 10^{-5}M ($3x10^{-8}$M in the tissue bath) –*6 min*
5. Ach 17 µl 10^{-4}M (10^{-7}M in the tissue bath) –*6 min*
6. Ach 51 µl 10^{-4}M ($3x10^{-7}$M in the tissue bath) –*6 min*
7. Ach 17 µl 10^{-3}M (10^{-6}M in the tissue bath) –*6 min*
8. Ach 51 µl 10^{-3}M ($3x10^{-6}$M in the tissue bath) –*6 min*

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