CHAPTER II
LITERATURE REVIEW

2.1 Hemodynamics

Homeostasis of hemodynamics refers to the regulation of the blood circulation to meet the demands of the different organ and tissue systems. The three parameters that involve the homeostasis of cardiovascular regulation are mean systemic arterial pressure (MAP), cardiac output (CO) and total systemic vascular resistance (SVR) in the circulation. Blood pressure depends on CO and resistance in the vessel; higher the CO and/ or resistance, will increase the arterial blood pressure (De Hert, 2012). Hence, the decreases of CO and/ or vascular resistance are considered for treatment of hypertension.

MAP = CO x SVR

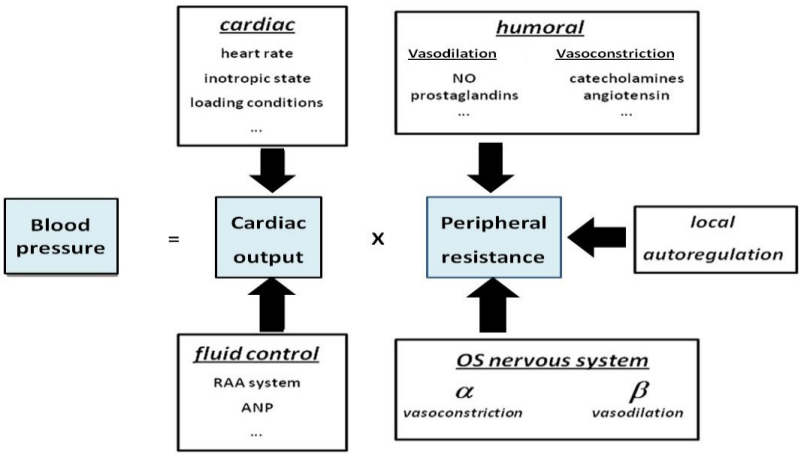

Figure 2.1 Overview of the different factors involved in the regulation of blood pressure. RAA, renin–angiotensin–aldosterone pathway; ANP, atrial natriuretic peptide; NO, nitric oxide; OS, orthosympathetic (De Hert, 2012)

The different factors involved in the regulation of blood pressure were concluded in Figure 1; there are cardiac control, humoral, local autoregulation, orthosympathetic and fluid control.

2.2 Hypertension

Hypertension refers to a blood pressure (BP) higher than normal range. It is one of the most common chronic diseases and contributors to morbidity and mortality in the word. It is estimated that about 20% of the world's adult population suffer from hypertension. The prevalence of hypertension increases in worldwide, approximately 40% of adults aged 25 and above had been diagnosed with hypertension; the number of people with the condition rose from 600 million in 1980 to 1 billion in 2008 (WHO, 2013). In Thailand, the prevalence of hypertension and prehypertension weighted to the national 2004 population from a nationally representative sample of 39,290 individuals aged ≥ 15 years was 22.0% and 32.8%, respectively, with a higher prevalence in men compared to women (Aekplakorn *et al.*, 2008). Remarkably, hypertension is the major risk factor of burden of disease (Bundhamcharoen *et al.*, 2011), the document from the Bureau of Epidemiology of the Ministry of Public Health of Thailand showed that the rate of mortality in Thai population in 2010-2012, (21,142 deaths per 100,000) from heart diseases and 3,684 deaths per 100,000 from hypertension (MOPH, 2013). The data suggest that prevalence of hypertension is rising rapidly, and spreads across regions in Thailand and all around the world. The increase in cardiovascular risk inherent to hypertension leads to premature morbidity and mortality. Prevention of blood pressure combined with lifestyle interventions or pharmacological treatments are associated with reductions in the risk of stroke and coronary events and appear to reduce to a lesser degree of the incidences of other complications (Williams, 2009).

Hypertension can be primary or essential, the etiology of which is unclear and thought to be resulting from the interaction of multiple genetic and environmental factors, and secondary to a defined process, such as renal artery stenosis. The pathophysiology of essential hypertension is heterogeneous and varies by age, renin status, sodium dependency, and other characteristics (Watson *et al.*, 2002). There are several experimental models have been developed to mimic the many aspects of human hypertension such as L-NAME hypertensive rats, spontaneously hypertensive rats (SHR) and 2-kidney, 1-clip (2K-1C) renovascular hypertension.

2.3 Endothelial function

The vascular endothelium is a monolayer of simple squamous cells, which is highly active endocrine organ covering the inner surface of the arteries and veins. Endothelium cell provides a large surface area to generate chemically mediated control of vascular homeostasis. The endothelium is an important regulator of vascular homeostasis especially, vascular tone via the release of relaxing factors such as NO, endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2). The most potent of vasodilator is now identified as NO (Furchgott and Zawadzki, 1980). Endogenous NO is synthesized from the metabolism of L-arginine through the action of the eNOS, whereas PGI_2 derived from arachinodic acid possibly produced through the cyclooxygenase pathway (Moncada *et al.*, 1991; Nagao and Vanhoutte, 1993). The gaseous molecule NO exerts its relaxing effect by diffusing towards the underlying vascular smooth muscle cell layer to dilate blood vessels in a cyclic guanylyl monophosphate-dependent manner. Whereas, PGI_2 induced vasodilation by increasing the production of cAMP (Gryglewski *et al.*, 1986) and leading to membrane hyperpolarization. NO can also diffuse towards the lumen to prevent platelet adhesion and activation, and also monocyte adhesion. NO prevents the expression of prothrombotic and proatherosclerotic mediators including tissue factor, the physiological activator of the coagulation cascade, adhesion molecules, chemo attractant factors and the oxidation of LDL (Figure 2). A prominent role exists for EDHF in the control of resistance artery tone by hyperpolarizing vascular smooth muscle. PGI2, generated by the arachidonic acid cascade via cyclo-oxygenases (COX), activates the cyclic AMP pathway during its vasodilator activity (Andriantsitohaina *et al.*, 2012). The endothelium synthesized and released NO in the response of chemical, neurohumoral, shear stress and physical stimuli. To maintain the vascular tone, NO counteracts the vasoconstrictive effects of norepinephrine, serotonin, vasopressin and angiotensin II (Ang II) by stimulating the receptors on the endothelium.

1) Nitric oxide (NO)

Endothelium-derived nitric oxide (or NO) is a paracrine factor. Vascular NO production can be stimulated by a variety of receptor agonists as well as the shear stress produced by the flowing blood. NO dilates all types of blood vessels by stimulating soluble guanylyl cyclase and increasing cGMP in smooth muscle cells. There are very important in regulation of vascular tone, inhibits platelet aggregation, prevents adhesion of leukocytes, reduces proliferation of the intima and, inhibits proliferation of vascular smooth muscle cells (Forstermann, 2008; Forstermann and Munzel, 2006). An increased inactivation and/or reduced synthesis of NO are seen in the risk factors for cardiovascular disease.

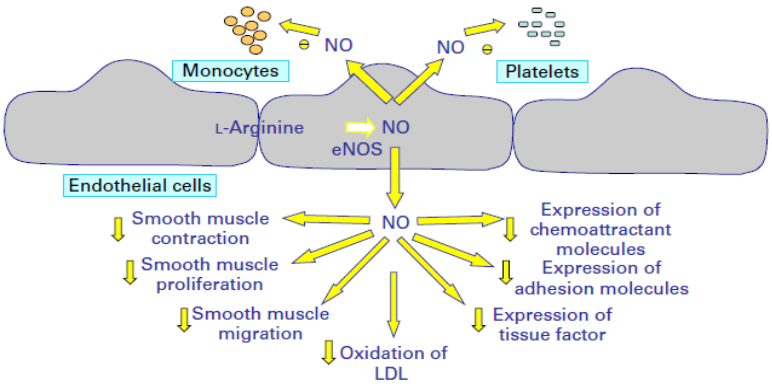

Figure 2.2 Endothelium-derived NO contributes to the regulation of vascular homeostasis. In healthy blood vessels, endothelial cells release NO, which is produced from L-arginine by endothelial NO synthase (eNOS). NO diffuses towards the underlying vascular smooth muscle to reduce vascular tone and keep smooth muscle cells in a non-migratory and non-proliferative state. NO can also diffuse towards the lumen where at the surface of endothelial cells, it prevents platelet adhesion and aggregation, and adhesion of monocytes. In addition, NO is also a potent inhibitor of the expression of several proatherothrombotic molecules such as tissue factor, chemoattractant molecules such as monocyte chemoattractant protein-1, and adhesion molecules such as vascular cell adhesion molecule-1. Moreover, NO retards the oxidation of LDL, a key step in the development of atherosclerosis (Andriantsitohaina *et al.*, 2012)

2) NO synthesis

NO is a simple diatomic gas, that it easily diffuse across cell membranes. NO derived from L-arginine (L-arg) by the action of a family of enzymes call NO synthases (NOS). NOS are expressed in a variety of tissue throughout the body, there are three type of NOS isoform, neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), an inducible NOS isoform expressed in a variety of activated tissues, and endothelial NOS isoform (eNOS or NOS III). The N terminus exhibits homology principally to the other NOS isoforms, whereas the C-terminal domain has significant sequence homology to cytochrome P450 reductases. The NOS N terminus binds tetrahydrobiopterin (BH_4) and heme (Figure 3). L-arginine binds in the enzyme active site near heme, while molecular oxygen is directed to the ferrous heme iron. The binding sites for BH_4 and heme are localized along the interface of the monooxygenase domains of the dimeric, active form of NOS. The C-terminal domain binds nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and Flavin mononucleotide (FMN) cofactors (Stuehr, 1997). The N- and C-terminal domains are linked by a short sequence that binds calmodulin (CaM), an allosteric effector that is essential for full NOS activity. The intact NOS functions as a mixed function monooxygenase/reductase enzyme, and has the capability to synthesize a number of molecules besides NO, most notably superoxide anions. In all NOS catalysis reactions, electrons are shuttled within the reductase domain sequentially from NADPH, to FAD, and finally to FMN. CaM is believed to function in facilitating the flow of electrons from both the reductase domain to the monooxygenase domain as well as from FAD to FMN. Because electrons appear to flow from the reductase domain of one NOS monomer to the oxygen- gase domain of another NOS monomer, enzyme dimerization is required for full enzymatic activity (Michel and Vanhoutte, 2010). Biosynthesis of NO involves a two-step oxidation of L-arginine to L-citrullinne (L-cit), with concomitant production of NO (Figure 2). The reaction consumes 1.5 mol of NADPH, and 2 mol of oxygen (O_2) per mol of L-citrulline formed. Initially, hydroxylation of L-arginine leads to the formation of N^G-hydroxyl-L-arginine, which can also act as a substrate for NOS. This is followed by oxidation of the intermediate, using a single electron from NADPH to form L-citrulline and NO (Andrew and Mayer, 1999; Griffith and Stuehr, 1995).

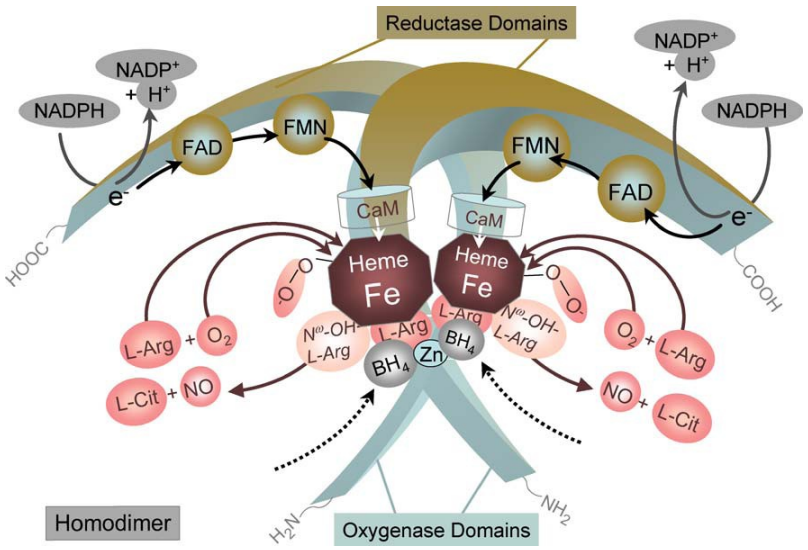

Figure 2.3 Basic structure of endothelial NO synthase (eNOS) and scheme of eNOS catalysis. All NOS enzymes are homodimers of two identical subunits. Each subunit consists of a reductase domain and an oxygenase domain. The flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) cofactors in the reductase domain accept electrons from nicotinamide adenine dinucleotide phosphate (NADPH) and pass them on to the heme in the oxygenase domain. When sufficient substrate L-arginine and cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH_4) are present, intact NOS dimers couple their heme and O2 reduction to the synthesis of NO. L-Citrulline is formed as the byproduct. N^W-Hydroxy-L-arginine (N^w-OH-L-Arg) is an intermediate in the reaction; NOS performs two separate oxidation steps, one to form N^w-hydroxy-L-arginine and a second to convert this intermediate to NO (Forstermann, 2010)

The main source of endothelial NO, a crucial factor for the normal functioning of the cardiovascular system is eNOS that expressed in endothelial cells. eNOS is constitutively expressed and located to caveolae in the plasma membrane. eNOS is usually activated by shear stress and or Ca^{2+}-mobilizing agonist promotes calcium-bound calmodulin binding to eNOS and caveolin-1 rapidly dissociates from the enzyme, rendering the enzyme active (Michel and Feron, 1997).

Caveolin-1 displacement may coincide with depalmitylation, and as a consequence of both these process, eNOS is released from the plasma membrane into cytosol. Activated eNOS generates NO until intracellular Ca^{2+} return to basal levels. Calmodulin dissociates from the enzymes and the inhibitory eNOS-caveolin complex reforms (Feron *et al.*, 1998). Therefore, NO production from endothelial cell is stimulated by factors that causing increasing intracellular Ca^{2+}concentration, including receptor- dependent agonist like acetylcholine, bradykinin, substance P, thrombin, and also shear stress, which is physical stimuli (Furchgott, 1983). The rate of NO production is determined by activity of NOS (Nishida *et al.*, 1992; Weiner *et al.*, 1994). NO production can be interrupted by many inhibitors in biological activity of NO. For instant, (i) the decreased in L-arginine uptake, (ii) decreased in co-factors ($Ca^{2+,}$ Calmodulin, and BH_4), (iii) inhibition of NOS expression, (vi) inhibition of electron flow (NADPH, FDH), (v) NO scavengers ,and (vi) oxygen-derived free radicals (Cai *et al.*, 2003; Moncada *et al.*, 1991).

Endothelium released NO play an importance role in vasodilation effects to VSMCs. NO is generated by conversion of the amino acid L-arginine to NO and L-citrulline by the enzyme nitric oxide synthase (NOS). The isoform NOSIII (eNOS) is constitutively expressed by the endothelium. NO exerts its relaxing effect on vascular smooth muscle by activation of soluble guanylate cyclase leading to initiates quanosine triphosphate (GTP) transformation to cyclic guanosine monophosphate (cGMP) and a reduction in intracellular calcium (Figure4) (Behrendt and Ganz, 2002).

Activation of cyclic GMP dependent on protein kinase G and followed by cytosolic Ca^{2+} removal from cell and inhibit contractile apparatus. Action of protein kinase G has a direct influence to phosphorylation of gap junctions, and also influences on potassium (K^+) and Ca^{2+} channels. Phosphorylation of K^+ channel causes K^+ outflow from cell, while phosphorylated Ca^{2+} channel decreases Ca^{2+} influx. When Ca^{2+} cytoplasmic concentration decreases below 500 nM, the phosphorylation will be stop. This happens because of Ca^{2+} unbinding with calmodulin, followed by detachment from myosin light chain kinase, causing its inactivation. Dephosphorylated myosin light chain prevents myosin head from binding to actin, resulting relaxation of smooth muscle (Ignarro and Kadowitz, 1985; Lincoln *et al.*, 1994).

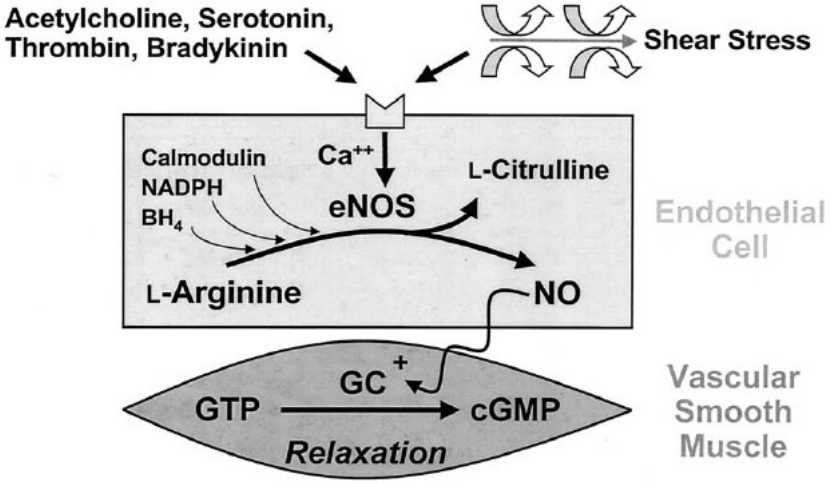

Figure 2.4 The nitric oxide signaling pathway (Behrendt and Ganz, 2002)

2.4 Endothelial dysfunction

Endothelial dysfunction is characterized by a shift of the actions of the endothelium toward reduced vasodilation, a proinflammatory state, increase platelet activation, thrombus formation, increased permeability, leukocyte adhesion and monocyte migration into the vascular wall, and impaired regulation of vascular growth and vascular remodeling (Endemann and Schiffrin, 2004). Moreover, the mechanisms that contribute in the reduced vasodilatory responses in endothelial dysfunction include reduced nitric oxide generation, up-regulation of adhesion molecules, generation of chemokines such as macrophage chemoattractant peptide-1, and production of plasminogen activator inhibitor-1 participate in the inflammatory response.

Increased ROS bioavailability and oxidative stress together with decreased NO production because of reduced eNOS activity and increased NO consumption by ROS, together with reduced production of hyperpolarizing factor are contributable to many of the molecular events underlying endothelial impairment.

Oxidative excess in hypertensive patients leads to diminished NO and correlates with the degree of impairment of endothelium-dependent vasodilation and with cardiovascular events (Heitzer *et al.*, 2001). Therefore, endothelial dysfunction is a hallmark for vascular diseases and is associated with various pathologies, such as hypertension, diabetes, atherosclerosis, ischemic heart disease and chronic kidney

disease (Hink *et al.*, 2001; Panza *et al.*, 1995). In animal models of hypertension, oxidative stress leads to endothelial dysfunction as evidenced by impairment of endothelium-dependent relaxation.

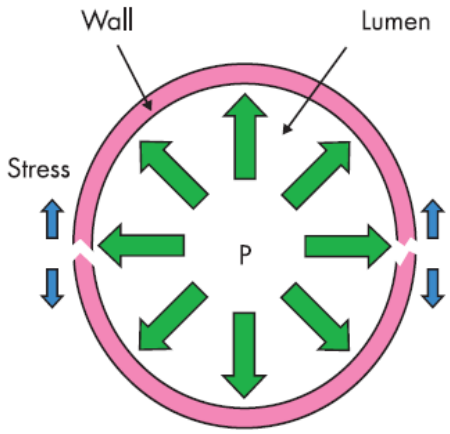

Figure 2.5 Wall stress and tension in a vascular wall (Laplace's law) (Mayet and Hughes, 2003)

2.5 Hypertensive vascular remodeling

Hypertension is greatly implicated in the development progression of structural and functional alteration in both resistance and conduit arteries, which increase peripheral resistance and compromise vascular compliance, respectively. Vascular remodeling is considered an adaptive response to elevation of arterial pressure to normalize the wall stress. A rise in tension results in increased wall tensile stress. Normalization of wall tensile stress can be achieved either by an increase in wall thickness or by a reduction in lumen diameter, or both (Mayet and Hughes, 2003)

The Laplace's law, tensile force in the vessel wall (T) depends on transmural pressure (P) and luminal radius (R) (equation 1). The wall stress (σ), force that applied to a specimen over a cross-sectional area (CSA), depends on the tensile force and the vascular thickness (h) (equation 2) (Figure 5).

Tensile force (T) = Pressure x Lumen redius (R) Equation 1

Wall stress = T/h Equation 2

Combining equation 1 and 2 give:

Wall stress = [P x R] / h Equation 3

1) General features of vascular remodeling

Subtype of vascular remodeling has been reported. It could be inward or outward, depending on whether the diameter (D) decreases or increases, and hypertrophic; eutrophic, or hypertrophic, if respectively contributed to by net growth (increase CSA), no net change in amount of tissue (constant CSA), or net loss of tissue (decrease CSA), (Figure 6) (Feihl *et al.*, 2008).

In resistance arteries (diameter < 300 μm - 15 μm) remodeling in essential or primary hypertension is associated with a reduced lumen and increased W/L ratio. This geometrical abnormality has been termed vascular inward remodeling. It has been proposed that resistance artery narrowing can be due to altered function, decreased distensibility or to modified wall structure. This alteration can occur with or without vessel growth; that is inward remodeling can be hypertrophic or eutrophic (Arribas *et al.*, 2006).

Hypertension is also associated with structural and mechanical alterations of conduit arteries. The main feature of large artery remodeling is wall hypertrophy around a normal or larger lumen size. Such structural changes necessary lead to an increased medial cross-sectional area, which may be mechanism to normalize wall tension, according to the Laplace's law (Feihl *et al.*, 2008). The changes in wall structure at the vascular smooth muscle cells can become hypertrophic (increase in cell volume with or without DNA polyploidy), hyperplasic (greater cell number), or both. In addition, the ECM components, especially collagen and elastin modification also contribute greater to the total wall mass.

Large arteries are known to affect mechanical performance, reducing the buffering function of large arteries. This, associates to the deleterious effects of hypertension by increasing pulse pressure, which is a strong predictor for coronary heart disease and myocardial infarction (Safar *et al.*, 2003). Remodeling of the vascular wall can be induced in response to long-lasting changes in arterial blood flow and/or pressure. The ultimate effect would be to maintain relatively constant tensile and/or shear stresses. This could explain the hypertrophic responses of the arterial

wall in some forms of hypertension. Furthermore, wall stress can also be normalized by reduce vascular lumen diameter. On the other hand, chronic decreases in blood flow reduce resistance artery diameter and wall mass (Arribas *et al.*, 2006).

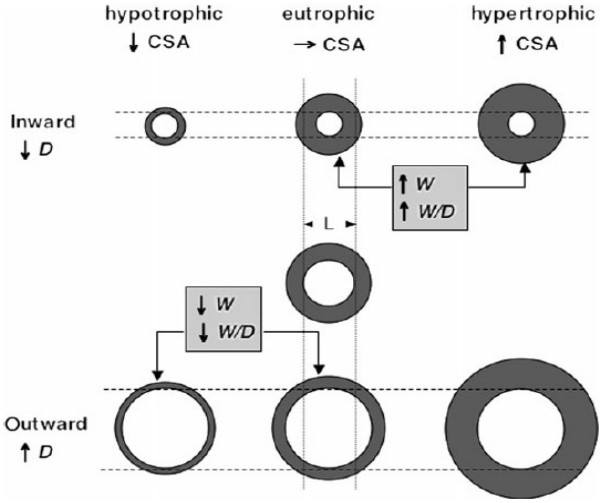

Figure 2.6 Subtypes of vascular remodeling. Each pair of concentric circles represents a vessel in cross-sectional view. Depicted in the centre of the figure is the reference state, with respect to which structural changes occur. CSA; cross-sectional area, D; lumen diameter, W; Wall thickness (Feihl *et al.*, 2008)

2) ECM alterations

The ECM of the vessel wall is composed of collagens, elastin, proteoglycans, and glycoproteins. Collagen and elastin are the major contents of ECM. The ECM also contain of a signals to endothelial cell and VSMCs. They are regulating the blood vessel formation and function during development and disease. Under the physiological conditions, the cyclic stretching of arterial wall sustains a quiescent, contractile VSMCs phenotype and slow turnover of ECM proteins. However, when the physical or chemical conditions changes or the arterial wall expose to the new environment by a process termed vascular remodeling, that the structure and function of the vessel wall are modified to accommodate to a new settings(Davis and Senger, 2008; Jarvelainen *et al.*, 2009).

Collagens and elastin constituents of arteries are increased in hypertensive patients and in genetic or experimentally induced hypertension in animals. The increase in collagen and elastin synthesis induces by the increased wall stress from high blood pressure. However, the synthesis of collagen and elastin returns to basal levels as soon as the blood pressure stops rising (Jacob *et al.*, 2001).

The biomechanical properties of vessels are dependent on the absolute and relative quantities of these compositions. The stiffness of arterial wall depends on balance of collagen and elastin. Collagen is about 100 times stiffer than elastin, and the gradual loss of elastin is inevitably accompanied by a reduction in vascular compliance (Martyn and Greenwald, 1997). Vascular smooth muscle cells respond to mechanical stretch by synthesis of collagen, with results in a thickening of the arterial wall and a further to loss of compliance (Leung *et al.*, 1977; Safar *et al.*, 1990). Thus, excessive collagen deposition and reduced elastin contents are the factor responsible for modified vascular stiffness in case of hypertension.

3) MMPs alterations

MMPs are a family of structurally related, zinc-containing enzymes that degrade the ECM and connective tissue proteins (Visse and Nagase, 2003). Increased the proteolytic effects of MMPs promotes vascular remodeling, excessive degradation of ECM, vascular smooth muscle cell migration and proliferation, and adhesion molecules (Galis and Khatri, 2002; Newby, 2006), for that reason MMPs activation contribute to vascular structural modification. The elevation of MMP-2 associated with vascular structural remodeling was found after chronic inhibition of NO synthesis (Bouvet *et al.*, 2005). Moreover, both MMP-2 and MMP-9 activity have been shown to increase in the vessel maintained at high pressure and MMP-9 has contributed to early hypertensive remodeling (Lehoux *et al.*, 2004). Furthermore, the role of MMPs mediated vascular changes in high blood pressure was confirmed by used of MMPs inhibitor (Guimaraes *et al.*, 2011). Increased expression and activities of several MMPs, especially MMP-2 and MMP-9 have also been reported in many vascular disease in human (Martinez *et al.*, 2006; Yasmin *et al.*, 2005). These suggested that, MMP-2 and MMP-9 may underlie in the vascular wall structure remodeling and involve the altering of ECM composition in hypertension. Various

studies are now focusing on the role of MMPs in the genesis and pathophysiology of hypertension.

2.6 Reactive oxygen species (ROS)

ROS are produced as intermediates in reduction-oxidation (redox) reactions leading from O_2 to H_2O. Of the ROS generated in endothelial cells, $O_2^{\bullet-}$ and H_2O_2 appear to be particularly important. In biological systems, $O_2^{\bullet-}$ is short-lived and unstable owing to its rapid reduction to H_2O_2 by superoxide dismutase (SOD), of which there are three mammalian isoforms, copper／zinc SOD (SOD1), mitochondrial SOD (SOD2) and extracellular SOD (SOD3) (Fridovich, 1997). The charge on the superoxide anion makes it unable to cross cellular membranes except possibly through ion channels. H_2O_2 has a longer lifespan than $O_2^{\bullet-}$, which is relatively stable and is easily diffusible within and between cells. The main source of H_2O_2 in vascular tissue is the dismutation of $O_2^{\bullet-}$.

$$2\ O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2^{\bullet}$$

This reaction can be spontaneous or it can be catalysed by SOD.

ROS are products of normal cellular metabolism and derive from many sources in different cellular compartments. The sources of ROS that are important in vascular disease and hypertension are xanthine oxidase, uncoupled nitric oxide synthase (NOS), and NADPH oxidase (Figure 7). NAD(P)H oxidase is a multisubunit enzyme, comprising gp91phox (or its homologs, Nox1 and Nox4), p22phox, p47phox (or NOXO1), p67phox (or NOXA1), and p40phox (Paravicini and Touyz, 2008).

1) Uncoupled eNOS

Under physiological conditions, NOS produces nitric oxide in the presence of co-factors L-arginine and tetrahydrobiopterin (BH_4). BH_4 is an essential co-factor for all three NOS isoforms, and all are capable of ‘uncoupling’. Basal NOS activity correlates with the amount of BH_4 bound to the enzyme. When BH_4 levels are reduced, the dimer structure is altered such that the oxidase domain yields molecular uncoupling and the catalytic activity becomes functionally ‘uncoupled’. Enzymatic

reduction of molecular oxygen by eNOS no longer couples to L-arginine, resulting in the generation of $O_2^{\bullet-}$ rather than NO. Endothelial NOS uncoupling, which shifts the nitrosoredox balance with adverse consequences, has been demonstrated in experimental models for instance, $O_2^{\bullet-}$ production was reduced in high-fat diet mice after pre-incubate with sepiapterin precursor of BH_4 (Ketonen *et al.*, 2010), eNOS uncouple in SHR was associated with reduced BH_4 content in aorta (Li *et al.*, 2006). Moreover, uncoupled eNOS has also been demonstrated in essential hypertension, in patients with hypercholesterolemia and in chronic smokers (Katusic *et al.*, 2009).

2) Xanthine oxidase

It is a key enzyme in the process of purine (e.g., xanthine and hypoxanthine) metabolism, which catalyzes the oxidation of hypoxanthine and xanthine to form $O_2^{\bullet-}$ is present in the vascular endothelium. Ischemia-reperfusion and hypoxia are condition that promote the accumulation of these substrate to form $O_2^{\bullet-}$ production and the increase in xanthine activity. $O_2^{\bullet-}$-derived from xanthine oxidase has been studied mainly in the context of cardiac disease, there is evidence suggesting involvement in vascular dysfunction in hypertension. For example, endothelial dysfunction in transgenic rats with overexpression of renin and angiotensinogen has also been associated with increased xanthine oxidase activity (Mervaala *et al.*, 2001), in experimental models of hypertension, xanthine oxidase activity is increased in the kidney. In addition, long-term inhibition of xanthine oxidase with allopurinol in SHR reduced renal xanthine oxidase activity without lowering blood pressure, indicating that the increased renal ROS production was a consequence of hypertension rather than a contributing factor (Laakso *et al.*, 1998).

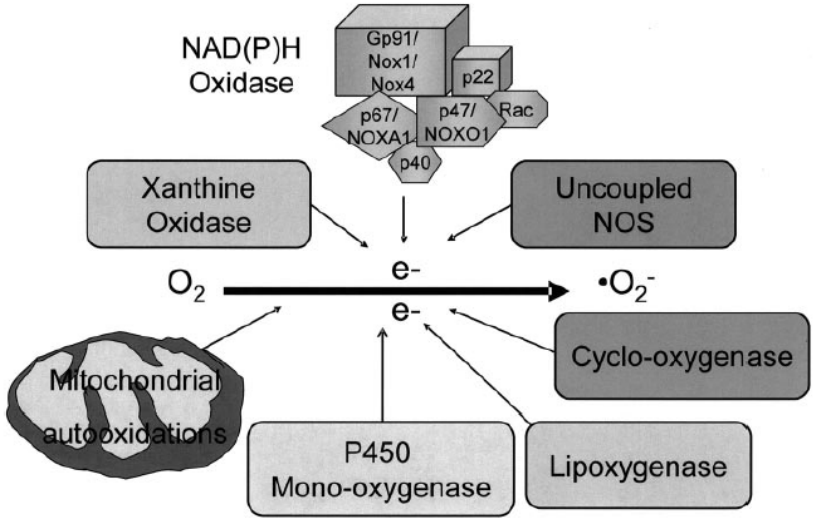

Figure 2.7 Enzymatic sources of superoxide anion ($O_2^{\bullet-}$). The major enzymes responsible for ROS generation in the vasculature include NAD(P)H oxidase, xanthine oxidase, and uncoupled NOS (Paravicini and Touyz, 2008)

3) NADPH oxidase

NADPH oxidase are a family of enzymes that generate ROS in mammalian cell types including endothelial cells and VSMCs. NADPH oxidase members, they catalyse the reduction of molecular oxygen to generate $O_2^{\bullet-}$ and /or H_2O_2 in varies intracellular and extracellular compartment. NADPH oxidase is composed of two membranes—bound elements gp91phox or Nox2 and p22phox, three cytosolic proteins: p40phox, p47phox, p67phox and a small G-protein Rac. They are activated by translocation of the cytosolic subunits p47phox, p67phox, and Rac to the Nox/p22phox complex.

The NADPH oxidase complex is an important source of superoxide generation in artery wall and mounting evidence suggests it associated with oxidative stress and endothelial dysfunction, in conditions such as hypertension, hypercholesterolaemia, diabetes and ageing. Many document suggested that, experimental models of hypertension such as, Ang II- induced hypertension, two-kidney, one-clip renovascular hypertensive rats fed with a high-fat, high-sucrose diet, SHR and chronic blockade of NO production was associated with increased

expression of NOX1, p^{22}phox or p^{47}phox NADPH oxidase subunit (Kong *et al.*, 2009; Sarr *et al.*, 2006; Yamashita *et al.*, 2007). Moreover, increased expression of NADPH oxidase subunit was associated with over production of ROS within arterial wall and endothelial dysfunction. Further supporting report, treatment with NADPH oxidase inhibitor in hypertensive rats reduced in vascular $O_2^{\bullet-}$ production, blood pressure and also improve endothelial function (Beswick *et al.*, 2001; Jimenez *et al.*, 2007; Jung *et al.*, 2004). Therefore, NADPH oxidase may be a major source of ROS generation and cause endothelial dysfunction in hypertensive rat model.

2.7 Oxidative stress and hypertension

Oxidative stress is defined as an excessive in the levels of oxidants over antioxidant within a biological system, and the direct consequence of this is a shift in the redox state of biological compartment -the DNA, mitochondria, lipid and protein- toward one that is more oxidizing and cause oxidative damage.

The relationships between oxidative stress and endothelial dysfunction have been demonstrated in many experimental models of hypertension. Study in isolated arteries from differences animal model of hypertension have shown to reduce endothelium-dependent relaxations to ACh, increased vascular reactivity to vasoconstrictors and also blunted to vasodilator in hypertensive patients (Taddei *et al*., 1998; (Perticone *et al.*, 2005). Oxidative stress contributes to the generation and/or maintenance of hypertension via a number of possible mechanisms. These include the overproduction of $O_2^{\bullet-}$, which in turn reduced vascular NO bioavailability. Excessive oxidative stress causes uncoupling eNOS which disrupts the eNOS dimer to generate eNOS monomer leading to generation of $O_2^{\bullet-}$ instead of NO (Yamashita *et al.*, 2007), and also promote cellular damage as indicated by increasing the levels of lipid per oxidation, protein oxidation and DNA damage as shown in several animal models of hypertension.

Several enzymes in the endothelial cell can produce $O_2^{\bullet-}$, the main source for oxidative excess in the vasculature is NADPH oxidase (Hamilton *et al.*, 2002). Other sources include xanthine oxidase (Landmesser *et al.*, 2002) and eNOS itself, when it is uncoupled by lack of substrate (L-arginine) or shortage of the co-factor tetrahydrobiopterin (BH4) (Cai and Harrison, 2000).The dominant mechanism of

impaired vascular NO bioavailability contributes to it $O_2^{\bullet-}$ scavenge NO with resulting formation of peroxinitrite ($ONOO^-$), a powerful oxidizer and causes intense cellular damage. Moreover, excessive production of $O_2^{\bullet-}$ and reduced NO are linked to the exaggerated oxidation , increases the release of the proinflammatory cytokines and growth factors, enhancing inflammatory process, changing vasopermeability, favoring endothelial adhesion of leukocytes that, in turn, interferes with the ability of endothelial cell- NO to relax vascular smooth muscle, thus causing vascular dysfunction (Zardi *et al.*, 2005).Hence, increased oxidative stress is considered a major mechanism involved in the pathogenesis of endothelial dysfunction.

In animal models of hypertension, oxidative excess leads to endothelial dysfunction as indicated by impaired endothelium-dependent relaxation. Oxidative excess in hypertensive patients leads to diminished NO (Taddei *et al.*, 1998) and correlates with the degree of impairment of endothelium-dependent vasodilation and with cardiovascular events (Heitzer *et al.*, 2001). Findings in animal models of renovascular hypertension suggest that enhanced generation of ROS leads to decrease NO bioavailability and endothelial dysfunction, which may be improved by antioxidant (Montenegro *et al.*, 2010). Moreover, in high-fat diet-induced obesity in mice, increased oxidative excess also led to endothelial dysfunction (Ketonen *et al.*, 2010). In addition, in patient with chronic renal failure, administration of vitamin C improved endothelial dysfunction of resistance arteries but not of conduit arteries (Cross *et al.*, 2003).

2.8 Animal models of hypertension

2.8.1 2K-1C renovascular hypertension

Renovascular hypertension is a clinical consequence of excessive stimulation of the renin–angiotensin–aldosterone system (RAAS). Obstruction of renal artery leads to a drop in perfusion pressure in juxtaglomerular apparatus, which reacts by increasing rennin secretion and a secondary increase in blood pressure. The release of renin activates a cascade system in which renin promotes the conversion of Ang I to Ang II and increases aldosterone release from the adrenal gland. Ang II causes severe vasoconstriction and aldosterone increases sodium and water retention, both causing an increase in BP.

The relationship between renal artery stenosis and hypertension was elegantly demonstrated by the work of Goldblatt and coworkers in 1934 (Goldblatt *et al.*, 1934). Goldblatt hypertension is induced in dogs by unilateral clamping of the renal artery. This was followed by similar production of hypertension in other species, i.e. rats. These models are classified as 2kidney-1clip (2K-1C), 2kidney-2clip (2K-2C), and 1kidney-1clip (1K-1C) hypertension models.

The 2K-1C is induced by placing a silver clip onto a renal artery. A unilateral stenosis of the renal artery reduces renal perfusion pressure and stimulates renin synthesis and release from the clipped kidney, while the nonclipped contralateral kidney is present.

The direct and indirect effects of the increased circulating Ang II concentrations along with the resultant increases in aldosterone production and the Ang II-dependent increases in the activity of the sympathetic nervous system contribute to the impaired excretory capability of the non-clipped kidney (Braam *et al.*, 1995). These interacting effects contribute greatly to the early developmental stages of 2K-1C Goldblatt hypertension, when plasma renin activity and circulating Ang II concentrations are elevated. As renal perfusion pressure to the clipped kidney is reestablished, however, the plasma renin activity and circulating Ang II concentrations return toward the normal range, whereas the arterial pressure remains elevated.

Previous study reported that, the development and maintenance of hypertension in 2K-1C hypertensive rat model is not merely due to the vasoconstrictor effects of Ang II released through activation of the RAAS, but also involves oxidative stress, endothelial dysfunction, and vascular remodeling.

In the past years, many contributions have evidenced oxidative stress involvement in the development of renovascular hypertension. NADPH oxidase is the most important source of $O_2^{\bullet-}$ production in the blood vessel, which is upregulated in many hypertensive condition especially Ang II (Zalba *et al.*, 2001). Ang II has been shown to stimulate the generation of $O_2^{\bullet-}$ in vascular tissue (Sarr *et al.*, 2006). Experimental evidence suggests that free radicals generated by Ang II specific mechanisms has the ability to scavenge endothelial NO so that reduced NO

bioavailability, impaired endothelium-dependent relaxation to ACh (Martinez *et al.*, 2008).

Recent studies have reported that a reduction in the production of endogenous NO, an activated biomolecule generated by nitric oxide synthase (NOS), has been implicated in many cardiovascular disorders, including renovascular hypertension. The impaired NO production has been demonstrated in angiotensin-II (Ang II)-induced and aorta constricted rats with left ventricular hypertrophy (LVH) (Wenzel *et al.*, 2007). Moreover, previous study have demonstrated phosphorylated eNOS, NOS activity, NO production and cGMP contents were markedly decreased in ventricular, aortas and left kidney tissues of 2K1C rats (Kong *et al.*, 2009; Li *et al.*, 2010; Wu *et al.*, 2012). Furthermore, the cellular accumulation of ROS caused lipid peroxidation, detected as plasma accumulation of TBARS (Garcia-Saura *et al.*, 2005). A consistent finding with reduced plasma nitrite and nitroso species levels (Montenegro *et al.*, 2010), and diminished antioxidant glutathione activity in 2K-1C hypertension (Mansour *et al.*, 2011).

Vascular remodeling characterize by degradation and reorganization of extracellular matrix in the vessel wall (Raffetto and Khalil, 2008). Vascular remodeling is an adaptive response to elevation of arterial pressure to normalize the wall tension (Safar *et al.*, 1998). Increased concentrations of ROS have been shown to play a role in the vascular changes associated with experimental renovascular hypertension, which characterize by increasing wall thickness, media/ lumen ratio and cross sectional area in both conduit and resistance arteries (Castro *et al.*, 2009). Vascular structural changes were associated with increased vascular smooth muscle cell number, excessive EMC (collagen and elastic contents) (Castro *et al.*, 2008) and promotes MMPs activation in vascular tissue (Castro *et al.*, 2009; Guimaraes *et al.*, 2011).

2.8.2 L-NAME-induced hypertension

The vascular endothelial cells synthesize NO by using eNOS. It has been demonstrated that administration of a NO synthase inhibitor, i.e. N^{ω}-nitro-L-arginine methyl ester (L-NAME), induced high blood pressure, increased peripheral vascular resistance, increased vascular $O_2^{\bullet-}$ production, reduced antioxidant glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities (Kitamoto *et*

al., 2000; Nakmareong *et al.*, 2011), and altered vascular structure and function (Bouvet *et al.*, 2005); (Nakmareong *et al.*, 2012). Chronic NO synthase inhibition in rats induced endothelial dysfunction and increased vascular responsiveness to adrenergic stimuli and inflammation (Gonzalez *et al.*, 2000; Holecyova *et al.*, 1996; Hsieh *et al.*, 2004; Torok and Gerova, 1996). It is found that other factors, including endothelial constricting factors (Paulis *et al.*, 2008), and renin-angiotensin system (Takemoto *et al.*, 1997) were also involved in L-NAME-induced hypertension. In addition, morphological studies in long term treatment with NO revealed vascular hypertrophy of both large and small arteries, increased MMP-2 expression in vascular tissue, and caused coronary atherosclerotic lesion (Bouvet *et al.*, 2005; Suda *et al.*, 2002).

NO-deficient hypertension may be reversed either by removing the initiating cause (ceasing of L-NAME administration) (Bernatova *et al.*, 2002) or by administration of various antihypertensive drugs. Interestingly, rats treated with various natural antioxidants have been reported to activate nitric oxide synthase and to increase the expression of NOS isoforms leading to blood pressure reduction and improvement of endothelial function (Kojsova *et al.*, 2006). Red wine polyphenols have been reported to possess beneficial properties for preventing cardiovascular disorders including NO-deficient hypertension in rats (Bernatova *et al.*, 2002; Jendekova *et al.*, 2006; Pechanova *et al.*, 2004)(Bernatova *et al.*, 2002; Jendekova *et al.*, 2006; Pechanova *et al.*, 2004). Red wine polyphenols prevented, at least in part, the elevation of blood pressure, aortic thickening and vascular dysfunction. Furthermore, a recent study by our group showed that, revealed curcumin and tatrahydrcurcumin, which are a natural antioxidant have also been reduced blood pressure, vascular stiffness but increased NO production, and increased antioxidant enzyme glutathione in this model (Nakmareong *et al.*, 2011; Nakmareong *et al.*, 2012). These beneficial changes were associated with an increase of NOS activity and reduction of oxidative stress in the aorta and left ventricle (Pechanova *et al.*, 2004). The imbalance between NO production and ROS formation was also suggested to be an important factor in several other forms of hypertension. Decreased NO bioavailability seems to be the principal factor responsible for the development of L-NAME-induced hypertension. Such an idea is also supported by the fact that this type

of hypertension can be prevented by NO donor administration. Therefore, research to date has focused mainly on the cardiovascular system in which reduced bioavailability of NO is target therapeutic option in the treatment of cardiovascular diseases.

2.9 Dietary antioxidants

2.9.1 ACE inhibiting peptides

Biologically active peptides or functional peptides are food derived peptides. These bioactive peptides are inactive within the original protein but, once released, function as regulatory compounds with hormone-like activity that is based on the inherent amino acid composition and sequence. In this regard, they may present active ingredients in functional foods and nutraceuticals. Numerous peptides exhibiting various activities have been reported, including opiate, mineral binding, immuno-modulatory, anti-thrombotic, antimicrobial peptides, antioxidative and antihypertensive effect with ACE inhibiting activity (Clare and Swaisgood, 2000; Korhonen and Pihlanto, 2003). Bioactive peptides usually contain two to twenty amino acid residues per molecule. These peptides can be liberated from the parent protein during gastrointestinal digestion in the body or during food processing (Clare and Swaisgood, 2000).

ACE plays an important role in the renin–angiotensin system, which regulates arterial blood pressure as well as salt and water balance. In the cardiovascular system, ACE converts angiotensin I to angiotensin II, a potent vasoconstrictor, and degrades bradykinin, a vasodilator. Therefore, inhibition of ACE, by ACE inhibiting drugs like captopril have been shown to result in an antihypertensive effect in patient hypertensive patients.

Many studies have focus on the ACE inhibiting peptides isolated from different foods protein. Frequently, ACE inhibiting activity is analyzed in *vitro* and implies the determination of the ACE activity by means of synthetic substrates with amino-substituted tri and dipeptide, such as hippuryl- L- histidyl-L-leucine (Nakamura *et al.*, 1995). Moreover, antihypertensive effect of ACE inhibiting peptide can be measured *in vivo* by oral administration in animal model of hypertension.

In recent years, foods derived peptides have been demonstrated to antihypertensive with ACE inhibitor both in *vitro* and in *vivo* study. For instant, single oral administration of dose 400 mg/kg body weight of peptides from whey protein reduced systolic blood pressure in SHR (Tavares *et al.*, 2012), hydrolysis of Few-flower wild rice (*Zizania latifolia* Turcz.) by proteases shown ACE inhibiting activity and free radical scavenging activity in vitro study. Long term supplement with a sardine peptides preparation, which was produce by hydrolysis of sardine muscle, has been found to reduced systolic blood pressure, tissue ACE activity and blood glucose in stroke-prone spontaneously hypertensive rat (Otani *et al.*, 2009).. In addition, after intravenous administration to SHR of the ACE inhibiting peptide isoleucine-valine-tyrosine, isolated from wheatgerm hydrolysate, the tripeptide is metabolized by the action of aminopeptidase in plasma to form a subsequent ACE inhibitor, valine-tyrosine. Valine-tyrosine exerts an acute depressor effect in SHR and the blood pressure returns to the normal state about 5 min after injection. On the other hand, after injection of isoleucine-valine- tyrosine, the reduction in blood pressure is much stronger and is held for 15 min. Therefore, it is suggested that the intake of isoleucine-valine-tyrosine as a physiologically functional food would serve in lowering blood pressure by the combined action of itself and its metabolite after absorption (Matsui and Matsumoto, 2006). Valine-tyrosine has been administered orally to mild hypertensive subjects. This suggests that bioactive peptides that possess ACE inhibiting activity may be useful of protection and treatment of hypertension.

2.9.2 Rice bran and rice bran protein hydrolysis

Rice (*Oryza sativa* L.) is the main staple food of the Asian population, and also the largest staple product exporter of Thailand. More than half of the production belongs to Asian countries such as China, Vietnam and Thailand. This large amount of production results in commensurate amount of rice by-products. One of the major by-products is rice bran which is produced during rice milling. Rice bran constitutes about 10% of rough rice grain and contains 18–22% oil. The proteins in rice bran are of a complex nature. Rice bran proteins contain 37% albumin, 36% globulin, 22% glutelin, and 5% prolamin (Sayre and Saunders, 1990). The oil containing oleic acid (38.4%), linoleic acid (34.4%) and linolenic acid (2.2%) as unsaturated fatty acids, and palmitic (21.5%) and stearic (2.9%) acids as saturated

fatty acids is generally extracted and used as high quality cooking oil. In addition, Sereewatthanawut and coworkers reported that extracts protein and amino acids from deoiled rice bran by subcritical water hydrolysis showed the protein content of the raw rice bran before oil extraction was 12%, the oil content was 16.75%, and the content of nonprotein (carbohydrate and ash) was 54%. After the oil was extracted, the oil content was 1.9% but the protein and non-protein contents found in deoiled rice bran increased to 19% and 58%, respectively (Sereewatthanawut *et al.*, 2008). Furthermore, the beneficial components such as sterols, higher alcohols, gamma-oryzanol, tocopherols, tocotrienols, vitamin B and phenolic compounds are found in rice bran (Min *et al.*, 2011; Zhang *et al.*, 2010).

Rice bran oil has a high nutritive value, offering benefits such as rice bran oil consumption decreased total serum cholesterol concentrations in patients with type 2 diabetes (Lai *et al.*, 2012). Oryzanol from rice bran has a greater effect on lowering plasma non- high-density lipoprotein cholesterol (HDL-C) levels and raising plasma HDL-C compared to ferulic acid (Wilson *et al.*, 2007). Beside the rice bran oil, rice bran protein hydrolysate has been investigated for antihypertensive effect on SHR (Li *et al.*, 2007). The alcalase-generated hydrolysate showed strong *in vitro* ACE inhibiting activity with the IC_{50} value of 0.14 mg/ml. A significant decreased in systolic blood pressure in SHR rats was observed following single oral administration of this hydrolysate at dosage of 600 mg/kg body weight (Li *et al.*, 2007). A potent ACE inhibiting peptide with the amino acid sequence of Thr-Gln-Val-Tyr (IC_{50}, 18.2 μM) was isolated and identified from the hydrolysate (Li *et al.*, 2007). A single dministration of Thr-Gln-Val-Tyr at 30 mg/kg body weight showed a significant decrease in systolic blood pressure in SHR (Li *et al.*, 2007). These results suggest that i*n vitro* ACE inhibiting activity and *in vivo* antihypertensive activity are presented in rice bran protein hydrosate. However, the mechanisms involved with this action are needed for further investigation. Interestingly, a recent study by Kokkaew and Thawornchinsombut showed that hydrolysate from Thai Hom-Mali rice bran possesses strong ACE inhibiting activity (Kokkaew and Thawornchinsombut, 2011).

2.9.3 Natural polyphenols

Diet, one of the lifestyle risk factors that strongly associated with the incidence of cardiovascular disease (Arts and Hollman, 2005). However, many

document from epidemiological studies suggest that regular intake of polyphenol-rich beverages and food such as red wine, tea, and fruit or vegetables is associated with an improved cardiovascular prognosis (Di Castelnuovo *et al.*, 2002; Mukamal *et al.*, 2002; Sofi *et al.*, 2008). The beneficial effects of polyphenols on the cardiovascular system have been reported to their ability to vascular relaxant effect, blood pressure lowering effects, reduce vascular oxidative stress by direct superoxide anion scavenging properties and interaction with other reactive oxygen species such as hydroxy and peroxy radicals (Nijveldt *et al.*, 2001) and their inhibitory effect on xanthine oxidase and NAD(P)H oxidase, which are major enzymes generating large amounts of reactive oxygen species (Orallo *et al.*, 2002). Moreover, to the antioxidant effects of polyphenols, both experimental and clinical studies purposed that polyphenols might also protect the cardiovascular system by improving the endothelial function.

Numerous studies indicate that polyphenols from various sources such as cocoa, tea, red wine, honey, propolis, curcumin, resveratrol and soy isoflavones can induce endothelium-dependent relaxations in the arteries (Anselm *et al.*, 2009; Karim *et al.*, 2000; Lorenz *et al.*, 2004; Martin *et al.*, 2002; Rakici *et al.*, 2005; Taubert *et al.*, 2002; Vera *et al.*, 2005; Xu *et al.*, 2007). In addition, antihypertensive effects of varieties polyphenols have been extensively studies, for instant intake of red wine polyphenol prevent angiotensin II-induced hypertension and endothelial dysfunction associated with decrease vascular $O_2^{\bullet-}$ production and prevent nox1 and p22phox NADPH oxidase subunits expression in angiotensin II infusion in rats (Sarr *et al.*, 2006). Moreover, phenolic compound extract from red wine, Azuki bean, black and green tea, blue burry have also been attenuated blood pressure in animal models of hypertension (Bernatova *et al.*, 2002; Mukai and Sato, 2009; Shaughnessy *et al.*, 2009).

2.9.4 Curcumin

The turmeric (*Curcuma longa* Linn.) plant, a perinial herb belonging to the ginger family (Zingiberaceae family), is cultivated extensively in south and southeast tropical Asia. It cans growth up to 1 m. high, oblong leaves, and pyriform or oblong rhizomes. There is often branched and brownish- yellow color. The rhizome or the root and is the most useful part of the plant for culinary and medicinal purposes

for centuries. The turmeric contains a wide variety of phytochemicals such as, curcumin, demethoxycurcumin, bidemethoxycurcumin, zingiberene, curcumenol, curcumol, eugenol, tetrahydrocurcumin, triethylcurcumin, turmerin, turmerones and, turmeronols. Interestingly, curcumin is an active gradient in turmeric, which may constitute 2 % to 8% of the turmeric and responsible for the bright yellow color (Aggarwal *et al.*, 2007; Epstein *et al.*, 2010).

Curcumin (diferuloymethane), is a natural polyphenol compound extracted from the rhizome of *Curcuma longa* Linn., which is a perennial tree widely cultivated in tropical regions of Asia. The turmeric has been used for centuries as a coloring and flavoring spice in foods and in folk medicine for treatment of variety of inflammation conditions and other diseases as well (Ammon and Wahl, 1991; Araujo and Leon, 2001). Curcuminiods is a yellow- pigmented fraction from turmeric that related to a principle ingredient of curcumin. Curcumin (CUR) is a member of the liner diarylheptanoid class of natural products in which two oxy-substituted aryl moieties are linked together through a seven-carbon chain. The major curcuminoids present in turmeric are CUR, demethoxycurcumin and bis-demethoxycurcumin. CUR is the most abundantly occurring natural analogue, followed by demethoxycurcumin in which one methoxy group is absent, then bis-demethoxylcurcumin in the methoxyl group is absent from both the aryl rings (Anand *et al.*, 2008).

Regarding Thai traditional medicine, fresh and dried rhizomes are used as peptic ulcer treatment, carminatives or wound treatment and anti-inflammatory agent (Wuthi-udomlert *et al.*, 2000). The Government Pharmaceutical Organization of Thailand (GPO) has developed many products by using CUR extracts for example, moisturizing efficacy of a commercial CUR extract cream (GPO CUR cream), CUR eye gel, and CUR lotion. Previous studies reported that CUR at high dose is safety, tolerability and non-toxicity both of animal model and human studies ((Lao *et al.*, 2006).

CUR has been studied in various animal models and clinical studies including anti-inflammatory, anticancer, anti-diabetic, anti-dementia, and antioxidant properties (Aggarwal and Sung, 2009; Rinwa *et al.*, 2010; Sharma *et al.*, 2005). CUR exerts its effective antioxidant activity through the ability to scavenge $O_2^{\bullet-}$ and hydroxyl radicals (OH•), and induction of antioxidant and cytoprotective enzymes

(Dinkova-Kostova and Talalay, 2008; Motterlini *et al.*, 2000; Somparn *et al.*, 2007). Treatment with CUR, a known activator of Nrf2-antioxidant response element (ARE) pathway, may suppress oxidant formation (Heiss *et al.*, 2009). Orally ingestion of CUR at dose of 3.6 g/day for 7days reduced the oxidative DNA adducts level, thereby lowering the risk for mutations and other genetic damage (Garcea *et al.*, 2005). Furthermore, CUR has protective effect against myocardium ischemia by inhibited effects on xanthine oxidase/xanthin dehydrogenase (XD/XO) conversion leading to decreased $O_2^{\bullet-}$ generation (Manikandan *et al.*, 2004). CUR also stimulates vasorelaxation of the isolated porcine coronary artery (Xu *et al.*, 2007). Moreover, we have recently reported the potential effect of CUR on prevention and treatment of vascular dysfunction in lipopolysaccharide-induced endotoxemia in mice (Sompamit *et al.*, 2009). In addition, CUR has been shown to prevent the blood pressure elevation, endothelial dysfunction and reduced oxidative stress in L-NAME hypertensive rats (Nakmareong *et al.*, 2011). These effects may associate with reduced vascular superoxide production and increased NO bioavailability as indicated by increasing lasma nitrate/ nitrite and prevention of down-regulation of eNOS (Ramaswami *et al.*, 2004).

2.10 Conceptual framework