ANTIOXIDANT STATUS AND THE EFFECT OF TOCOTRIENOL-RICH-FRACTION (TRF) SUPPLEMENTATION ON THE ANTIOXIDANT ENZYMES AND DEVELOPMENT OF HYPERTENSION IN SPONTANEOUSLY HYPERTENSIVE RATS (SHR)

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2010

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BY

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THESIS SUBMITTED IN FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

(MEDICAL PHYSIOLOGY)

UNIVERSITI SAINS MALAYSIA

JUNE 2010

ACKNOWLEDGEMENTS

First and foremost, I would like to thank the almighty GOD for his abundant benevolence that has brought me this far. May his kindness and benevolence be showered on people of all faiths, always. I would like to express my deepest gratitude and thanks to my supervisors, Prof Dr Harbindar Jeet Singh and Assoc Prof Dr KNS Sirajudeen for their kind and dedicated supervision throughout my studies. I am also thankful to Mr Chandran Govindasamy who taught me how to perform the biochemical analysis and to Assoc Prof Dr Shaharum Shamsuddin and Prof Dr Ravichandaran Manikam for their guidance on the PCR analysis. I would like to thank Dr Che Badariah, Head of Department of Physiology, for her continuous support during my studies. My special thanks also to all the staff of Department of Physiology, Laboratory Animal House Unit and Central Research Lab for their kind assistance throughout the period of my study. I would also like to take this opportunity to express my sincere thanks to all my family members and friends for their continuous encouragements and guidance throughout the duration of my study.

I would like to acknowledge the Ministry of Higher Education for the financial support for this research work via the Fundamental Research Grant Scheme (FRGS) (203/PPSP/6170021). I also would like to acknowledge the Ministry of Science, Technology and Innovation for giving me a scholarship (National Science Fellowship) for study. Finally, I would like to acknowledge the Institute of Postgraduate Studies, USM for their financial support that permitted me to attend qPCR Conference and Workshop in Freising, Germany early this year.

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LIST OF ABBREVIATIONS USED

ANOVA:	analysis of variance
AOE:	antioxidant enzymes
AP-1:	activator protein-1
ATP:	adenosine triphosphate
b:	base
bp:	base pairs
CAT:	catalase
CD:	cardiovascular diseases
cDNA:	complementary DNA
CD36:	cluster of differentiation-36
cGMP:	cyclic guanosine monophosphate
C_T:	threshold cycle
CuZn-SOD:	copper/zinc- superoxide dismutase
DEPC:	diethylpyrocarbonate
DNA:	deoxyribonucleic acid
dNTP:	deoxyribonucleotide triphosphate
DOCA:	desoxycorticosterone acetate
dR:	fluorescence value
dsDNA:	double-stranded DNA
DTNB:	5, 5’-dithiobis (2-nitrobenzoic acid)
Ec-SOD:	extracellular- superoxide dismutase
EGF:	epidermal growth factor
EGFR:	epidermal growth factor receptor

eNOS:	endothelial nitric oxide synthase
FAK:	focal adhesion kinase
GPx:	glutathione peroxidase
GPx-1:	cytsolic GPx
GR:	glutathione reductase
GSH:	reduced glutathione
GSSG:	oxidized glutathione
GST:	glutathione *S*-transferase
GSTA4:	glutathione *S*-transferase alpha 4
GSTM1:	glutathione *S*-transferase mu 1
GATT1:	glutathione *S*-transferase theta 1
H_2O:	water
H_2O_2:	hydrogen peroxide
HPLC:	high performance liquid chromatography
IGF-1:	insulin growth factor-1
IGF-1R:	insulin growth factor-1 receptor
IU:	international unit
JAK2:	janus kinase 2
kDA:	kilo dalton
LDL:	low density lipoprotein
MAPK:	mitogen-activated protein kinases
MBF:	medullary blood flow
MDA:	malondialdehyde
min:	minute
mmHg:	millimeters mercury

MMPs: metalloproteinases

M-MuLV: moloney- murine leukemia virus

Mn-SOD: manganese- superoxide dismutase

mRNA: messenger ribonucleic acid

NAD^+: nicotinamide adenine dinucleotide

$NADP^+$: nicotinamide adenine dinucleotide phosphate

NADPH: reduced form of $NADP^+$

NF-κB: nuclear factor-kappa B

$NO/NO^{\bullet}$: nitric oxide

NOS: nitric oxide synthase

O_2 : oxygen

$O_2^{\bullet -}$: superoxide anion

$^{\bullet}OH$: hydroxyl radical

PCR: polymerase chain reaction

PDGF: platelet derived growth factor

PDGFR: platelet derived growth factor receptor

PI3-K: phosphoinositide 3-kinases

PKC: protein kinase C

PPM: parts per million

PTK: protein tyrosine kinases

PTP: protein tyrosine phosphatases

PUFA: polyunsaturated fatty acids

RBC: red blood cell

RNA: ribonucleic acid

RNS: reactive nitrogen species

ROS:	reactive oxygen species
RPM:	revolutions per minute
rRNA:	ribosomal RNA
RT-PCR:	real-time polymerase chain reaction
SGLT-1:	sodium dependent glucose cotransporters-1
SHR:	spontaneously hypertensive rats
SOD:	superoxide dismutase
SOD-1:	copper/zinc- superoxide dismutase
SOD-2:	manganase- superoxide dismutase
SOD-3:	extracellular- superoxide dismutase
SP-SHR:	stroke prone- spontaneously hypertensive rats
SSA:	sulfosalicylic acid
TAS:	total anti-oxidant status
TBA:	thiobarbituric acid
TBARS:	thiobarbituric acid reactive substances
TBE:	tris base boric acid ethylenediaminetetracetic acid
TNF-α:	tumor necrosis factor-α
TP:	total protein
TRF:	tocotrienol-rich-fraction
tRNA:	transfer RNA
TTP	tocopherol transport protein
UV:	ultra violet
VLDL:	very low density lipoprotein
VSMC:	vascular smooth muscle cells
WKY:	Wistar-Kyoto

α:	alpha
β:	beta
δ/Δ:	delta
γ:	gamma

LIST OF PRESENTATIONS

Sirajudeen KNS, **Arunkumar S**, Lee SK, Singh HJ. Oxidative stress markers and the effect of tocotrienol rich fraction supplementation in spontaneously hypertensive rats. *Presented in International Conference on Advances in Free radicals, Natural products, Antioxidants and Radioprotection in Health & 9th Annual Meeting of the Society of Free radical Research, January 2010, Hyderabad, India.*

Arunkumar S, SK Lee, KNS Sirajudeen, HJ Singh. Free radicals, Antioxidant Enzymes and Redox Signaling. Emphasis on Hypertension. *Presented in Seminar at Dept of Health Sciences, April 2009, University of Genoa, Italy.*

Arunkumar S, SK Lee, KNS Sirajudeen, HJ Singh. Comparison of AOE activities and expression levels in the kidney during the development of hypertension in SHR. *Presented in 4th International qPCR Symposium and Workshop, March 2009, Technical University of Munich, Germany.*

Arunkumar S, Lee SK, Sirajudeen KNS, Singh HJ. Age-related changes in superoxide dismutase in the kidney of spontaneously hypertensive rats and normotensive rats. *Presented in 13th National Conference on Medical Sciences, May 2008, USM, Malaysia.*

Arunkumar S, Lee Siew Keah, KNS Sirajudeen, HJ Singh. Age-related changes in antioxidant enzyme status in the kidneys of spontaneously hypertensive rats and normotensive rats. *Presented in 22nd MSPP Meeting, Mac 2008, UM, Malaysia.*

STATUS ANTIOKSIDAN DAN KESAN PEMBERIAN PECAHAN YANG KAYA DENGAN TOKOTRIENOL (TRF) KE ATAS ENZIM ANTIOKSIDAN DAN PERKEMBANGAN HIPERTENSI DALAM TIKUS HIPERTENSI SPONTAN (SHR)

ABSTRAK

Walaupun stress oksidatif telah dikaitkan dengan patogenesis hipertensi dalam tikus hipertensi spontan (SHR), status sebenar enzim antioksidan (AOE) dan beberapa parameter yang berkaitan seperti glutation total (GSH), status antioksidan total (TAS), malondialdehid (MDA), serta tahap α-, δ- dan γ- tokotrienol plasma semasa perkembangan hipertensi masih tidak jelas. Oleh itu, kajian ini memeriksa parameter tersebut di ginjal SHR dari umur 2 hingga 16 minggu, dan kesan pemberian pecahan yang kaya dengan tokotrienol (TRF) terhadap parameter tersebut serta perkembangan tekanan darah tinggi dalam spesis ini.

Berbanding dengan tikus WKY yang berpadanan umur, aktiviti katalas (CAT) lebih tinggi secara signifikan seawal umur 2 minggu, manakala aktiviti glutation peroksida (GPx) lebih rendah secara signifikan bermula dari umur 4 minggu dalam SHR. Ekspresi mRNA juga menunjukkan perubahan yang serupa dengan aktivitinya. Tahap TAS lebih tinggi secara signifikan dalam SHR pada umur 4, 10, 12 dan 16 minggu, manakala tiada perbezaan pada tahap MDA kecuali pada umur 16 minggu apabila tahapnya lebih tinggi dalam SHR. Tahap plasma α-tokotrienol lebih rendah secara signifikan sejak umur 6 minggu manakala tahap γ-tokotrienol lebih rendah secara signifikan pada umur 12 dan 16 minggu dalam SHR.

Berbanding dengan SHR yang berpadanan umur dan tidak diberi TRF, tahap plasma α-, γ- dan δ- tokotrienol lebih tinggi secara signifikan dalam SHR yang diberi TRF. Purata tekanan darah sistolik lebih rendah secara signifikan pada umur 6 minggu dalam SHR yang diberi TRF, tetapi lebih tinggi secara signifikan pada umur 12 dan 16 minggu. Aktiviti glutation reduktas (GR) di ginjal lebih tinggi pada umur 6, 8 dan 10 minggu, dan aktiviti glutation *S*-transferas (GST) di ginjal juga lebih tinggi pada umur 6, 10 dan 12 minggu dalam SHR yang diberi TRF. Tahap MDA secara konsisten lebih rendah dalam SHR yang diberi TRF. Pemberian TRF kepada tikus WKY tidak memberi kesan ke atas tekanan darah atau aktiviti AOE primer kecuali aktiviti dan tahap ekspresi mRNA superoksida dismutas (SOD) yang lebih rendah dalam SHR yang diberi TRF. Walaubagaimanapun, aktiviti GR adalah lebih tinggi secara signifikan pada umur 6, 8 dan 10 minggu dalam tikus WKY yang diberi TRF.

Kesimpulannya, oleh kerana terdapat perubahan ekspresi dan aktiviti sebilangan AOE sejak umur 2 minggu iaitu sebelum peningkatan sebenar tekanan darah berlaku, berkemungkinan perubahan tersebut menyumbang kepada perkembangan hipertensi dalam SHR. Bagaimana perubahan tersebut menyumbang kepada perkembangan hipertensi memerlukan kajian lanjut, terutamanya apabila tiada perbezaan ketara dalam tahap MDA dan TAS di tikus WKY dan SHR. Kesan pemberian TRF selama 12 minggu tidak dapat menghalang peningkatan tekanan darah atau mengubah aktiviti AOE primer di ginjal, walaupun terdapat penurunan tahap MDA dan peningkatan tahap TAS. Kajian menggunakan dos TRF yang lebih tinggi atau pecahan TRF secara individu atau bersama-sama dengan antioksidan

yang lain perlu dijalankan untuk memastikan peranan TRF dalam menurunkan tekanan darah tinggi.

ABSTRACT

Although oxidative stress has been implicated in the pathogenesis of hypertension in spontaneously hypertensive rats (SHR), the precise status of antioxidant enzymes (AOE) and a number of other related parameters such as total glutathione (GSH), total anti-oxidant status (TAS) and malondialdehyde (MDA), and plasma levels of α-, δ- and γ- tocotrienol during the development of hypertension remains unclear. This study therefore examined the levels of these in the kidneys of SHR from the age of 2 to 16 weeks, and the effects of tocotrienol-rich-fraction (TRF) supplementation on these and the development of high blood pressure in this species.

Compared to aged-matched WKY rats, catalase (CAT) activity was significantly higher from 2 weeks of age, and gluthathione peroxidase (GPx) activity was significantly lower from 4 weeks of age in SHR. A similar pattern was also evident in the mRNA expressions. TAS levels were significantly higher in 4-, 10-, 12-, and 16-week old SHR while MDA was unchanged except in 16-week old SHR where it was significantly higher. Plasma α-tocotrienol levels were significantly lower from the age of 6 weeks and onwards, while plasma γ-tocotrienol levels were significantly lower at 12- and 16-week old in SHR.

Compared to age-matched non-supplemented SHR, plasma α-, γ- and δ-tocotrienol levels were significantly higher following TRF supplementation in SHR. Mean systolic blood pressure was significantly lower at 6 weeks of age in TRF-supplemented SHR, but was significantly higher at 12 and 16 weeks. Glutathione reductase (GR) and glutathione *S*-transferase (GST) activities in the kidneys were

higher following TRF supplementation in 6, 8 and 10, and 6, 10 and 12-week old SHR respectively. TRF supplementation did not alter the activities of the primary AOE in the SHR. MDA levels were consistently lower in TRF-supplemented SHR. TRF supplementation to WKY rats had no effect on blood pressure or the activities of primary AOE except superoxide dismutase (SOD) activity and its mRNA levels, which were lower in TRF-supplemented WKY rats. However, GR activity was significantly higher in 6, 8 and 10-week old TRF-supplemented WKY rats.

In conclusions, the expression and activities of some of the AOE are altered in SHR, from as early as 2 weeks of age, and since these become evident before the actual rise in blood pressure it might be inferred that these alterations in some way contribute to the development of hypertension in the SHR. Precisely how these contribute to the rise in blood pressure needs to be examined further, particularly when no obvious differences were evident in the levels of MDA and TAS between WKY rats and SHR. TRF supplementation for 12 weeks neither prevents the rise in blood pressure nor significantly alters the kidney primary AOE activities and this despite of decreases in the levels of MDA and increases in TAS. Studies using higher doses of TRF or its individual fractions or in combination with other antioxidants need to be conducted to better ascertain if TRF has a role in reducing high blood pressure.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Free Radicals

Free radicals are defined as molecules or molecular fragments containing one or more molecular unpaired electrons in atomic or molecular orbits (Droge, 2002). This unpaired electron gives a considerable degree of reactivity to the free radicals. The biologically important free radicals have been divided into either reactive oxygen species (ROS) or reactive nitrogen species (RNS) (Droge, 2002). ROS includes superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), hydroperoxyl radical ($HO_2^{\bullet}$), peroxyl radical ($RO_2^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), hypochlorite ($HOCL^{-}$) and hypochlorus acid (HOCL). RNS includes nitric oxide ($NO^{\bullet}$/NO), nitrogen dioxide ($NO_2^{\bullet}$), peroxynitrite ($ONOO^{-}$), peroxynitrous acid (ONOOH), alkyl peroxynitrite (ROONO) and nitrothiols (RSNO).

ROS and RNS serve important cellular functions, for example, ROS is involved in stimulating several redox-sensitive cellular signaling pathways such as opening of ion transport systems, activation of protein phosphorylation and gene expressions (Figure 1.1 & 1.2) (Paravicini and Touyz, 2006, Rojas *et al.*, 2006, Valko *et al.*, 2007). These redox-sensitive cellular pathways play important roles in the modulation of vascular cell functions such as cell growth, apoptosis, migration, angiogenesis and cell adhesions (Yung *et al.*, 2006, Papaharalambus and Griendling, 2007). ROS regulates transcriptional factors such as nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) (Sen and Packer, 1996, Rojas *et al.*, 2006). NF-κB and AP-1 induce expression of pro-inflammatory genes, including monocyte

chemotactic protein-1 (MCP-1), adhesion molecules, and interleukins, that plays important roles in vascular inflammation, growth, and atherogenic process associated with chronic diseases such as hypertension and atherosclerosis (Brigelius-Flohe *et al.*, 2004). $NO^{\bullet}$ is one of the most widespread signaling molecules, and participates in numerous cellular functions (Ignarro *et al.*, 1999). Physiologic levels of $NO^{\bullet}$ produced by endothelial cells are essential for regulating the relaxation and proliferation of vascular smooth muscle cells, leukocyte adhesion, platelet aggregation, angiogenesis, thrombosis, vascular tone, and hemodynamics (Ignarro *et al.*, 1999).

ROS has multiple ways of interacting with processes linked to the various protein tyrosine phosphorylation-phosphatase systems (Torres, 2003, Chen and Keaney, 2004). The redox processes markedly influence the balance of activities between various mitogen-activated protein kinases (MAPK) in vessel wall, such as p42/p44 MAPK, stress-activated or c-Jun N-terminal kinase, and the p38 MAPK associated pathways. ROS may inhibit activity of tyrosine phosphatases, resulting in increased tyrosine phosphorylation, which influences oxidative stress-induced activation of receptor protein tyrosine kinases, such as the epidermal growth factor receptor (EGFR), insulin growth factor-1 receptor (IGF-1R), and platelet derived growth factor receptor (PDGFR). It also influences non-receptor tyrosine kinases, such as Janus kinase 2 (JAK2), focal adhesion kinase (FAK), phosphoinositide 3-kinases (PI3-K), all of which have been implicated in cardiovascular remodeling and vascular damage (Griendling *et al.*, 2000, Kamata *et al.*, 2000, Droge, 2002).

ROS can also modify critical residues in proteins, thereby altering their structures and functions. Oxidative modification of amino acid residues may occur in several ways and one such most described modification involves cysteine residues (Thannickal and Fanburg, 1995). Hydroxyl group of cysteine can be oxidized to form sulfenic (-SOH), sulfinic ($-SO_2H$), sulfonic ($-SO_3H$) or glutathionylated (-SGH) derivatives. This modification may lower the activity of an enzyme if amino acid residues being affected are located in the catalytic domain (Nakashima *et al.*, 2005) or decrease the ability of a transcription factor to bind to DNA if the corresponding DNA-binding domain is affected (Abate *et al.*, 1990).

ROS is also known to affect calcium channels (Waring, 2005). H_2O_2 has been proposed to act as an endothelium-derived hyperpolarizing factor (EDHF) that is released by the endothelium to cause vasodilatation by opening potassium channels in vascular smooth muscle (Sobey *et al.*, 1997, Miura *et al.*, 2003). Higher levels of H_2O_2 have also been shown to cause calcium-dependent release of NO from the endothelium (Yang *et al.*, 1999).

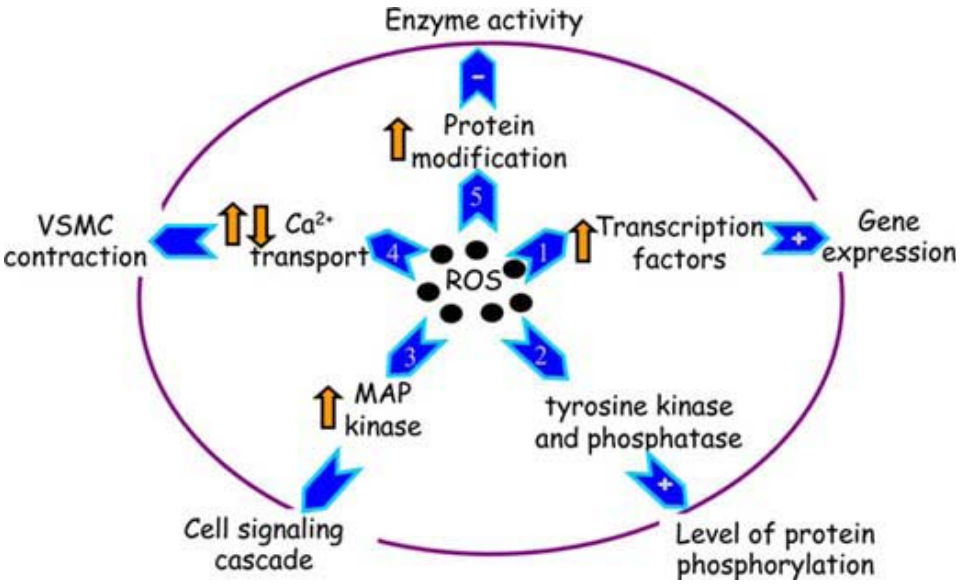


Figure 1.1 ROS-targeted signaling molecules (adapted from Yung *et al.*, 2006).

NOTE: (1) ROS regulate activities of several transcription factors, such as, nuclear factor kappa B (NF-κB), activator protein-1 (AP-1) and hypoxia-inducible factor-1 (HIF-1), which are involved in the regulation of the gene expression. (2) ROS regulate activities of protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP), which are required in maintaining controlled levels of protein phosphorylation. Tyrosine phosphorylation may be increased when cells are exposed to oxidant, due to PTP inactivation. (3) Mitogen-activated protein kinase (MAPK) is another target for ROS, which is a family of ubiquitous proline-directed, proteinserine/threonine kinases, participating in signal transduction classically associated with cell differentiation, growth and death. (4) ROS participate in calcium homeostasis by regulating calcium transport system. For example, peroxynitrite inhibits the activity of the sacroplasmic reticulum Ca^{2+}-ATPase and H_2O_2 activates L-type voltage-gated Ca^{2+} channels in SHR. (5) ROS are involved in the modification of proteins by interacting with crucial amino acid residues, so as to alter the formation of inter- and intra-molecular bonding, which finally affects the conformation and activity of proteins, such as protein kinase C (PKC) isoenzymes.

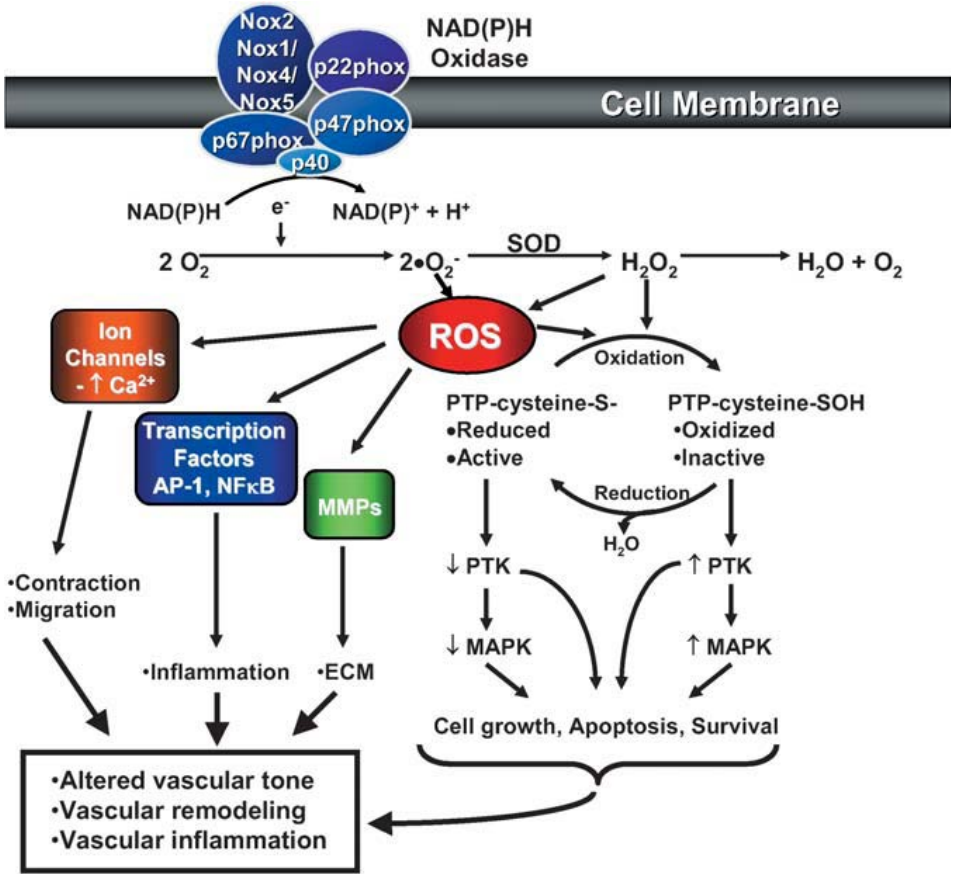


Figure 1.2 Redox-dependent signaling pathways in vascular cells (adapted from Touyz and Schiffrin, 2004).

NOTE: Intracellular ROS influence the activity of protein tyrosine phosphatases (PTP) by modifying cysteine residues. Oxidation of the cysteine residue to sulfenic acid by H_2O_2 renders PTPs inactive, whereas reduction renders PTPs active. Activated PTP decreases activity of protein tyrosine kinases (PTK) and mitogen-activated kinases (MAPK), whereas inactivated PTP have opposite actions. ROS also influence gene and protein expression by activating transcription factors, such as NF-κB and AP-1. ROS stimulate ion channels, such as plasma membrane Ca^{2+} and K^+ channels, leading to changes in cation concentration and matrix metalloproteinases (MMPs), which influence extracellular matrix proteins (ECM) degradation. Activation of these redox-sensitive pathways results in many cellular responses, which if uncontrolled, could contribute to altered vascular tone, increased vascular smooth muscle cells (VSMC) growth, inflammation and increased deposition of extracellular matrix protein, leading to vascular remodeling in hypertension. Decreased effect **(↓)** and increased effect **(↑)**.

Under physiological condition, the rate of generation of ROS or RNS appears to be balanced with the status of the antioxidant defense or *vice versa*. An imbalance between ROS or RNS generation and the antioxidants in favour of the former either through excessive production of ROS or RNS, or deficient antioxidant function, or both, has been ascribed as oxidative or nitrosative stress respectively (Droge, 2002, Valko *et al.*, 2007). During oxidative or nitrosative stress, the excess or non-neutralised free radicals may interact with a variety of macromolecules including lipids, proteins, and nucleic acids resulting in cell dysfunction (Droge, 2002). In addition, most free radicals often have a short half-life ranging from a fraction of a second to few seconds (Table 1.1). The cytotoxic effect of free radicals seems to be a critical mediator for many chronic vascular diseases such as hypertension and atherosclerosis (Dhalla *et al.*, 2000, Berry *et al.*, 2001). Thus, there is now a believe that there are "two faces" of free radicals in biology in that at physiological levels they serve as signaling and regulatory molecules but at high or pathological levels they are highly deleterious and cytotoxic oxidants (Valko *et al.*, 2004, Valko *et al.*, 2007).

Table 1.1 Half-life of different types of free radical (Devasagayam *et al.*, 2004).

Species	Symbol	Half-life at 37^0C/second
Superoxide	$O_2^{\bullet -}$	1×10^{-6}
Hydroxyl	$^{\bullet}OH$	1×10^{-9}
Peroxyl	$ROO^{\bullet}$	1
Hydrogen peroxide	H_2O_2	stable
Nitric oxide	$NO^{\bullet}$	1
Peroxynitrite	$ONOO^-$	1×10^{-3}

1.2 Biological Sources of ROS/RNS

ROS/RNS are derived from various biological sources including, mitochondrial electron transport chain, peroxisome, respiratory burst and enzymatic sources such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, endothelial nitric oxide synthase (eNOS) and myeloperoxidase.

Mitochondria generate approximately 2-3 nmol of superoxide/min per mg protein. During mitochondrial respiration, electron transport involves a coordinated four electron reduction of oxygen to water in the electron transport chain. However a small number of electrons "leak" prematurely from the mitochondrial respiratory chain enzymes forming the oxygen free radical superoxide (McIntyre *et al.*, 1999, Cadenas and Davies, 2000). Measurements of sub-mitochondrial particles suggest an upper limit of 1-3% of all electrons in the transport chain "leaking" to generate superoxide (McIntyre *et al.*, 1999, Cadenas and Davies, 2000).

Respiratory burst is a defense mechanism against invading organisms. In this process, activated macrophage, monocytes, neutrophils and eosinophils produce superoxide, hydrogen peroxide, hydroxyl and hypochlorite to kill microorganisms or to inactivate viruses (Halliwell, 1997a). Activation of cell membrane associated NADPH oxidase is involved in the generation of superoxide by reducing molecular oxygen during respiratory burst.

Peroxisomes are major sites of oxygen consumption in the cell and participate in metabolic functions that utilize oxygen (Valko *et al.*, 2004). High oxygen

consumption leads to H_2O_2 production, which is then used to oxidize a variety of molecules. Additionally, H_2O_2 is generated in the peroxisome by degradation of long-chain fatty acids by fatty acyl-CoA oxidase (Conway *et al.*, 1987, Schrader and Fahimi, 2004). When peroxisomes are damaged and their H_2O_2 consuming enzymes are down-regulated, H_2O_2 is released into the cytosol, which contributes significantly to cell damage associated with oxidative stress.

NADPH oxidase, which was originally found in the phagocytes, is a major source of $O_2^{\bullet-}$ in endothelial and VSMC (Griendling *et al.*, 2000). NADPH consists of the membrane subunits gp91phox and p22phox and the cytosolic subunits p40phox, p47phox, p67phox and GTPase rac1. NADPH oxidase is up-regulated by various stimuli including humoral factors such as angiotensin ll, platelet derived growth factor, tumor growth factor-α and hemodynamic forces including laminar and oscillatory shear stress (Touyz and Schiffrin, 2004).

eNOS is a cytochrome p450 reductase-like enzyme that catalyzes flavin-mediated electron transport from the electron donor NADPH to a prosthetic heme group (Cai and Harrison, 2000, Schulz *et al.*, 2004). The enzyme requires tetrahydrobiopterin, bound to a nearby heme group, to transfer electron to L-arginine to form NO. In the absence of either tetrahydrobiopterin or L-arginine, eNOS can produce $O_2^{\bullet-}$ and H_2O_2 thereby promoting oxidative stress. This phenomenon has been referred to as NOS uncoupling and uncoupling of eNOS diminishes NO production and therefore reduces vasodilation (Cai and Harrison, 2000). Additionally, eNOS can also become partially uncoupled, such that both $O_2^{\bullet-}$ and

NO are produced simultaneously resulting in the formation of peroxynitrite (Cai and Harrison, 2000). Under this condition, eNOS may become a peroxynitrite generator.

Xanthine oxidoreductase catalyses the oxidation of hypoxanthine and xanthine during purine metabolism (Cai and Harrison, 2000, Schulz *et al.*, 2004). Xanthine oxidoreductase exists in two forms, either as xanthine dehydrogenase or as xanthine oxidase. The former reduces nicotinamide adenine dinucleotide (NAD^+), whereas the latter prefers molecular oxygen, leading to the production of both $O_2^{\bullet-}$ and H_2O_2. This enzyme is not only expressed within cells but also circulates in the plasma and binds to endothelial cell extracellular matrix. Under pathological conditions, the enzyme is capable of producing large amounts of ROS.

Myeloperoxidase is a heme-protein expressed in neutrophils and monocytes, and secreted during activation of these cells (Wassmann *et al.*, 2004). Myeloperoxidase uses H_2O_2 to produce hypochlorus acid in a 2 step reaction involving the redox reaction intermediate compound I (a ferryl/porphyrin radical cation). The enzyme also exerts classic peroxide activity and oxidizes a variety of organic substrates to reactive oxygen intermediates involving compound I and compound II (a ferryl/protein radical species) formations. The important substrates are tyrosine and nitrite, resulting in the formation of tyrosyl radicals and nitrogen dioxide radicals.

1.3 ROS and RNS

$O_2^{\bullet-}$ is primarily produced as a free radical and interacts with other molecules to generate secondary ROS, either directly or through enzymes or metal catalyzed processes (Valko *et al.*, 2005). $O_2^{\bullet-}$ has a short half life of 10^{-6} seconds at 37°C (Devasagayam *et al.*, 2004). $O_2^{\bullet-}$ is not membrane permeable and therefore its site of reaction is limited to the compartment where it is produced (McIntyre *et al.*, 1999). $O_2^{\bullet-}$ is reduced into H_2O_2 by superoxide dismutase (SOD).

H_2O_2 is the non-radical family of ROS as it does not have an unpaired electron (Gutteridge, 1995). Some of the biological functions of H_2O_2 are activation of genes especially those controlled by NF-κB (Sen and Packer, 1996) and induction of intracellular calcium (Waring, 2005). Unlike $O_2^{\bullet-}$, H_2O_2 is membrane permeable and therefore its site of reaction can be different from its site of production. H_2O_2 is reduced by transient metal ions such as copper and iron to produce hydroxyl radical (Young and Woodside, 2001). Redox state of a cell is largely linked to iron (and sometimes copper) redox couple and is maintained within strict physiological limits (Valko *et al.*, 2005). It has been suggested that iron regulation ensures that there is no free intracellular iron. However under oxidative stress, excess superoxide acts as an oxidant to release free iron from iron containing molecules ($Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$). The released iron reacts with H_2O_2 in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$), generating ${}^{\bullet}OH$ (Young and Woodside, 2001). In addition, H_2O_2 also reacts with $O_2^{\bullet-}$ in the Haber-Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$), producing ${}^{\bullet}OH$ (Haber and Weiss, 1934). Hydroxyl radical is a highly reactive substance with a half life of less than 10^{-9} seconds (Devasagayam *et al.*, 2004). Due to its very short half-life, hydroxyl radical reacts close to its *in-vivo* site of formation.

It interacts with a variety of cellular macromolecules such as protein, lipid, carbohydrates and DNA resulting in their damage and subsequent deterioration of cell function (Reiter *et al.*, 2000).

Hypochlorous acid (HOCl) is formed by activated neutrophils during respiratory burst (Gutteridge, 1995). The heme-containing enzyme myeloperoxidase present in the phagocyte cytoplasm catalyzes the formation of HOCl from H_2O_2 and chloride ions. HOCl may also give rise to hydroxyl ion by an iron independent or iron dependent reaction (Candeias *et al.*, 1993).

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$$

$$HOCl + O_2^{\bullet -} \rightarrow {}^{\bullet}OH + Cl^- + O_2$$

$$HOCl + Fe^{2+} \rightarrow {}^{\bullet}OH + Cl^- + Fe^{3+}$$

The most commonly produced RNS is $NO^{\bullet}$. $NO^{\bullet}$ is a small molecule containing one unpaired electron on the anti-bonding to $2\pi^*_{\gamma}$ orbital. It is a biologically important signaling molecule in several physiological processes, such as neurotransmission, blood pressure regulation and in immune defense system (Alderton *et al.*, 2001). $NO^{\bullet}$ has a half life of only a few seconds in an aqueous solution (Chiueh, 1999). $NO^{\bullet}$ is membrane lipid soluble and readily diffuses through the cytoplasm and plasma membrane. It interacts with $O_2^{\bullet -}$ to produce peroxynitrite anion (Halliwell, 1997b). The interaction between $NO^{\bullet}$ and $O_2^{\bullet -}$ ($NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^-$) occurs at an extremely rapid rate (6.7×10^9 $M^{-1}s^{-1}$) (Thomson *et al.*, 1995). The reaction is three times faster than the reaction rate of $O_2^{\bullet -}$ with SOD (Thomson *et al.*, 1995). Peroxynitrite is a deleterious oxidative molecule that can cause DNA fragmentation, lipid and protein oxidation (Halliwell, 1997b). Peroxynitrite reacts

with tyrosine residues in various proteins to produce nitrotyrosin**e**. Alternatively, ROS can activate tyrosine residues to produce tyrosyl radicals that in turn can oxidize $NO^{\bullet}$ to produce nitrotyrosine (Eiserich *et al.*, 1995).

In conclusion, both ROS and RNS are highly reactive substances with very short half life and with important physiological roles. Due to their very short half-life, their reactions are usually close to their *in-vivo* site of formation. At physiological levels, ROS and RNS are involved in numerous cellular functions. However when present in excess they could cause serious deleterious effects to the cell structure and function. This is often referred to as oxidative stress and is believed to underly many disease processes. For optimal cell function, a stable level of ROS and RNS at the cellular level is maintained by the antioxidant enzyme and non-enzyme systems.

1.4 Antioxidant Enzymes

Antioxidant enzymes (AOE) constitute and represent an important part of the total antioxidant activity of aerobic cells. The primary AOE in cells include SOD, glutathione peroxidase (GPx) and catalase (CAT) (Yu, 1994, Gutteridge, 1995). Each of these AOE is known to perform detoxification of a particular ROS. SOD catalyses the conversion of $O_2^{\bullet -}$ to H_2O_2. CAT and GPx catalyse the decomposition of H_2O_2 into water. GPx also removes lipid peroxides by conversion of reduced glutathione (GSH) to oxidized (GSSG). Glutathione reductase (GR) regenerates GSH from GSSG for reuptake into the GPx reactions.

SOD is an endogenously produced antioxidant enzyme present in every cell in the body (Young and Woodside, 2001) and forms a major cellular defense system

against $O_2^{\bullet-}$. These enzymes contain redox metals (Cu^{2+} or Mn^{3+}) in the catalytic center and dismutate the superoxide radicals to hydrogen peroxide and oxygen ($2O_2^{\bullet-} + 2H^+ + SOD \longrightarrow H_2O_2 + O_2$). Three different isoforms of SOD have been identified, namely; the cytosolic copper/zinc-containing SOD (CuZn-SOD, SOD-1) (Groner *et al.*, 1985), the mitochondrial manganese-containing SOD (Mn-SOD, SOD-2) (Wan *et al.*, 1994), and the extracellular copper/zinc-containing SOD (Ec-SOD, SOD-3) (Folz and Crapo, 1994).

CuZn-SOD is composed of two identical subunits of about 32 kDA in which each subunit containing one copper and one zinc atom is bridged by a histamine residue at its active site (McCord and Fridovich, 1969, Mates *et al.*, 1999). The human gene for CuZn-SOD has been localized to the 21q22.1 region of chromosome 21 and spans around 11 kilo base pairs (bp) (Groner *et al.*, 1985). CuZn-SOD is principally located in the cytosolic, nucleic and lysosomal fractions of the cell. CuZn-SOD is believed to play a major role as a first line antioxidant defense system and contributes to the most of total SOD activity (Mates *et al.*, 1999).

. Mn-SOD is a homotetramer (96kDa) containing one manganese atom per subunit (Weisiger and Fridovich, 1973). The human gene for Mn-SOD has been localized to the 6q25 region of chromosome 6 and spans around 11 kilo bp (Wan *et al.*, 1994). Mn-SOD is found in the mitochondrial fraction of the cell, where it plays a central role in catalyzing superoxide anion generated within the mithochondria. Mn-SOD is synthesized in the cytoplasm and directed into the mitochondria by cell signaling peptides (McIntyre *et al.*, 1999).

Ec-SOD is a homotetramer (130kDa) in which each subunit contains one copper and one zinc atom (Marklund, 1982). The human gene for Ec-SOD has been localized to chromosome 4 and spans around 5.9 kilo bp (Folz and Crapo, 1994). It is generally produced by fibroblasts, glial and VSMC and secreted into the extracellular fluid. In the vasculature, it is found bound to the surface of endothelial cells (Marklund, 1982). Ec-SOD plays an important role in the regulation of oxidative balance in the vascular interstitium by neutralizing superoxide anion.

In general, the SOD iso-enzymes are hydrophobic and are mainly localized within cell compartments (Groner *et al.*, 1985, Wan *et al.*, 1994, Folz and Crapo, 1994) although, to some extent, SOD-3 is also found in the extracellular fluid (Marklund, 1982). SOD-1 and SOD-3 show 50% amino acid homology (Hjalmarsson *et al.*, 1987), particularly at the catalytic site (Tainer *et al.*, 1982), but neither shows any homology to SOD-2. SOD-1 and SOD-3 activity is inhibited by cyanide, which helps to distinguish these enzymes from SOD-2 (Borders and Fridovich, 1985). Enzymatic activities of SOD-1 and SOD-2 in catalyzing the dismutation of superoxide anions have similar efficiencies (Powers and Sen, 2000). However enzymatic reaction of SOD-3 against superoxide is reported to be higher than that of SOD-1 and SOD-2.

Superoxide dismutation ends with the production of a harmful byproduct known as H_2O_2. To this end, GPx and CAT are frequently used by cells to rapidly catalyze the decomposition of H_2O_2 into less reactive molecules (Yu, 1994, Gutteridge, 1995).

GPx catalyzes the reduction of H_2O_2 and lipid peroxides to water and lipid alcohols, utilizing reducing equivalents from GSH in which it is oxidized into GSSG (Young and Woodside, 2001).

$$H_2O_2 + 2GSH \xrightarrow[GPx]{} GSSG + 2H_2O$$

$$ROOH + 2GSH \xrightarrow[GPx]{} GSSG + ROH + H_2O$$

GPx is a homotetramer (80 kDa) with one selenocysteine residue in each monomer (Forstrom *et al.*, 1978). It requires selenium in the form of selenocysteine at the active site for its catalytic function. GPx is located in the membrane, nucleas, mitochondria, cytosol and other organelles of cells (Powers and Sen, 2000). The fact that GPx is distributed well, allows it to rapidly remove deleterious oxidant substances from a variety of cell sources. This characteristic makes GPx an important cellular protectant against ROS mediated damage to membrane lipids, proteins and nucleic acids. Five isoforms of this enzyme have been identified. They are cytsolic GPx (Flohe *et al.*, 1973), phospholipid hydroperoxide GPx (Ursini *et al.*, 1982), plasma form of GPx (Takahashi and Cohen, 1986), gastrointestinal form of GPx (Chu *et al.*, 1993), and non-selenium dependent GPx (Fisher *et al.*, 1999). Cytosolic GPx is present predominantly in erythrocytes, kidney and liver, and phospholipid hydroperoxide GPx is highly expressed in renal epithelial cells and testes (Mates *et al.*, 1999). Plasma form of GPx is also believed to be produced by

the kidney (Roxborough *et al.*, 1999). All GPx isoforms reduce H_2O_2 and alkyl hydroperoxides but differ in their specificities against hydroperoxide (Mates *et al.*, 1999).

CAT catalyzes the decomposition of H_2O_2 to water and oxygen molecules (Mates *et al.*, 1999). CAT is a tetramer with a molecular weight of (240 kDa) and each subunit contains a haem group and NADPH molecule (Mates *et al.*, 1999). It needs iron as a cofactor bound to the active site for its catalytic activity.

$$2H_2O_2 \xrightarrow[CAT]{} 2H_20 + O_2$$

CAT has one of the highest turnover rates of all enzymes; one molecule of CAT can convert millions of molecules of H_2O_2 to water and oxygen per second (Powers and Sen, 2000). By removing H_2O_2, it indirectly detoxifies $O_2^{\bullet-}$, which have been turned into H_2O_2 by SOD. CAT is an intracellular AOE that is largely located in cellular peroxisomes and to some extent in the cytosol and intracellular granules (Young and Woodside, 2001). Thus, CAT mainly decompensates H_2O_2, which is produced within the peroxisome. Under physiological conditions, peroxisome is known to produce H_2O_2, but not $O_2^{\bullet-}$ (Valko *et al.*, 2004). Higher oxygen consumption in the peroxisome leads to H_2O_2 production. The high content of CAT in this organelle prevents the accumulation of this toxic compound. However, when CAT activity is insufficient, H_2O_2 generated within peroxisome will be released into the cytosol, which then contributes to oxidative stress.

Although there is some overlap between the functions of CAT and GPx, the two enzymes differ in their affinity for H_2O_2. Mammalian GPx has a greater affinity for H_2O_2 at lower concentrations compared to CAT (Powers and Sen, 2000). This means at low steady state substrate concentrations, GPx plays a more prominent role in removing H_2O_2, and at high substrate concentrations, CAT plays a greater effective role in removing H_2O_2 (Gutteridge, 1995). Therefore, GPx is thought to be a major defense mechanism in low level of oxidative stress and CAT is effective in high level of oxidative stress or in the case of limited GPx reaction in protecting cells from H_2O_2 (Wassmann *et al.*, 2004). In the absence of adequate activity of these enzymes, H_2O_2 and lipid peroxides are converted to hydroxyl radicals and lipid peroxyl radicals, respectively, by transition metals (Gutteridge, 1995). Hydroxyl radical is a highly toxic radical and damage sustained as a consequence of free radicals is often largely caused by the devastating effect of hydroxyl radical. Unlike H_2O_2, hydroxyl radical is not enzymatically detoxified and membrane impermeable, thus it interacts with macromolecules within its site of generation and the radical damage is often referred as site specific (Reiter *et al.*, 2000).

GR is a dimer of two identical subunits of molecular mass 50 kD each (Gutterer *et al.*, 1999). GR requires NADPH as coenzyme for its catalytic activity and possesses one flavin molecule at its catalytic site. GR catalyses the reduction of GSSG to GSH using NADPH as an electron donor (Fridovich, 1978). The ratio of GSH to GSSG is kept very high by the enzymatic activity of GR and a low ratio of GSH/GSSH is an index that indicates tissue oxidative damage.

GST has a molecule weight of 45 kD and is dissociable into two subunits of approximately 25 kD (Habig *et al.*, 1974). Four classes of GST have been identified including alpha (α), mu (μ), pi (π) and theta (θ). (Board *et al.*, 1990). GST is a selenium-independent enzyme and it accepts a broad spectrum of organic hydroperoxides as substrates except H_2O_2 (Burk *et al.*, 1978). GST plays a major role in the detoxification process by catalyzing the conjugation of GSH to various electrophilic substrates (Habig *et al.*, 1974).

The activities of AOE are closely dependent to each another and coordination of their functions results in the maintenance of ROS below critical levels compatible with cell viability (Droge, 2002). These enzymes are tightly regulated in maintining a steady redox state to keep the entire system in a fully functioning state. Any disturbance of this tightly balanced system would lead to promotion of oxidation.

In addition to that, oxidative status of the cell may regulate the gene expression and activity of these AOE (Franco *et al.*, 1999, Rodriguez *et al.*, 2004). Moderate amount of radical stress stimulates the AOE reaction as an adaptive mechanism (Gechev *et al.*, 2002). For instant, total SOD, Mn-SOD, GPx and CAT activities were reported to be increased by two fold in the renal tissue following oxidant injury (Yoshioka *et al.*, 1990). However, exposure to greatly elevated oxidative stress on the other hand may reduce the enzymatic activities by inactivating or inducing degradation of these enzymes (Pigeolet *et al.*, 1990, Pigeolet and Remacle, 1991, Wei and Lee, 2002). High concentration of substrates including H_2O_2 and hydroperoxides have been found to inhibit GPx activity by 50 % while CAT activity was inactivated by $^{\bullet}OH$ and $O_2^{\bullet -}$, and SOD activity was inhibited by

H_2O_2 (Pigeolet *et al.*, 1990). In certain circumstances, the alteration of these AOE during oxidative stress is insufficient to neutralize the harmful effects of free radicals (Droge, 2002). For example, excessive production of $O_2^{\bullet -}$ may elevate SOD activity as a compensatory mechanism. However, induction of one enzyme may not necessarily lead to the induction of the other enzymes (Amstad *et al.*, 1991) e.g. in the case of mitigated GPx/CAT activity. Through a negative feedback mechanism, increasing amount of H_2O_2 will slowly inactivate SOD activity. Eventually, a deficiency or failure in the up-regulation of AOE activities will enhance oxidative stress resulting in tissue oxidant injury.

In summary, to counteract the deleterious effects of free radicals and other ROS or RNS, all cells are endowed with their own antioxidant systems that help contain the free radical activity within normal range. The levels of these AOE are tuned to match the production of free radicals. Any disturbance in this matching results in significant deleterious effects on the cells.

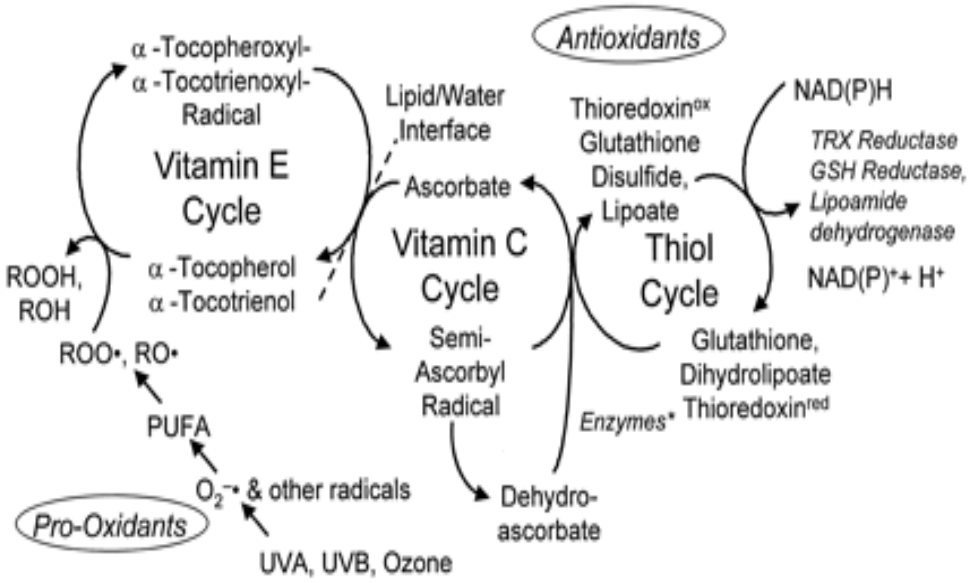
1.5 Vitamin E (Tocotrienol)

Vitamin E includes 8 different isoforms, the tocopherols (α, β, δ, λ) and the tocotrienols (α, β, δ, λ) (Schaffer *et al.*, 2005). The tocotrienol and tocopherol share a similar aromatic chromonal nucleus/head and a hydrocarbon side chain. The various isoforms of vitamin E (α, β, δ, λ) differ only in their methyl substituents on the chromonal nucleas. The difference between tocotrienols and tocopherols is in their corresponding aliphatic tails. Tocopherols have a saturated phytol chain, whereas tocotrienols have an unsaturated isoprenoid chain with double bonds at 3, 7 and 11 positions of the hydrocarbon tail (Schaffer *et al.*, 2005).

Vitamin E is incorporated into cellular membranes, where it inhibits the peroxidation of lipids by scavenging the chain propagating peroxyl radicals (Packer *et al.,* 2001). Vitamin E is the primary chain breaking antioxidant in cell membranes. Because of its high lipid solubility, it is associated with lipid rich membranes such as those of the mitochondria, sarcoplasmic reticulum and plasma membrane. The hydroxyl group of the vitamin E gives up its hydrogen atom to peroxyl radical and converts it to lipid peroxide and stops the propagation of lipid free radicals. Hence, vitamin E is a chain breaking antioxidant, it breaks the chain reaction of lipid peroxidation but itself gets converted into a radical during the process. Vitamin E consumed in this scavenging process is regenerated by vitamin C (Figure 1.3) (Packer *et al.*, 2001).

Antioxidant properties of tocotrienol in scavenging free radicals were found to be higher than the tocopherols. α-tocotrienol possesses 40-60 times higher antioxidant activity against (Fe^{2+} & NADPH) induced lipid peroxidation in rat liver microsomal membranes than α-tocopherol (Serbinova *et al.*, 1991). α-tocotrienol protection of cytochrome P450 againts oxidative damage was 6.5 times greater than α–tocopherol (Serbinova *et al.*, 1991). α-tocotrienol also exhibits significantly greater peroxyl scavenging potency than α-tocopherol in phosphatidylcholine liposomes (Suzuki *et al.*, 1993). It is suggested that antioxidant potency of α-tocotrienol requires a membrane environment, as incidently both antioxidants have identical activity when estimated in hexane *in-vitro* (Suzuki *et al.*, 1993). The superior antioxidant activity of tocotrienols is due to its higher recycling efficiency of chromanoxyl radicals and a uniform distribution and stronger disordering in

membrane bilayers, which makes the interaction between chromanols and lipid radicals more efficient (Serbinova *et al.*, 1991).



***** 1) Thiol transferase (glutaredoxin)
2) Glutathione (GSH)- dependent dehydroascorbate reductase
3) Protein disulfide isomerase
4) Thioredoxin (TRX) reductase

Figure 1.3 The antioxidant network showing the interaction among vitamin E, vitamin C and thiol redox cycles (Packer *et al.*, 2001).

1.6 Total Anti-oxidant Status

Total anti-oxidant status (TAS) indicates the capacity of both enzymatic (such as CuZn-SOD, Mn-SOD, CAT, GPx, GST, thioredoxin reductase and cytochrome oxidase) and non-enzymatic antioxidants (such as vitamin E, β-carotene, ubiquinol, transferrin, lactoferrin, albumin, haptoglobins, GSH, bilirubin, uric acid and vitamin C) to scavenge free radicals in biological samples (Gutteridge, 1995, Mates *et al.*, 1999, Young and Woodside, 2001). Antioxidant protections can occur at several different levels within cells, some of which include preventing free radical formation, intercepting radicals when formed, repairing oxidative damage caused by radicals, increasing the elimination of damaged molecules, and not repairing excessively damaged molecules to minimize the introduction of mutations (Gutteridge, 1995, Mates *et al.*, 1999, Young and Woodside, 2001).

1.7 Oxidative Stress & Hypertension

An imbalance between free radical production and antioxidant activity, often referred to as oxidative stress, has been implicated in some cardiovascular disease states like hypertension (Diaz *et al.*, 1997, Vaziri and Rodriguez-Iturbe, 2006, Ceriello, 2008). Elevated levels of markers of oxidative stress including, 8-deoxyguanosine, lipid peroxidation and GSSG/GSH ratio have been reported in patients with essential hypertension (Redon *et al.*, 2003, Abdilla *et al.*, 2007, Rodrigo *et al.*, 2007). The activities of SOD, CAT and GPx have also been found to be significantly decreased in whole blood and peripheral mononuclear cells of hypertensive individuals (Redon *et al.*, 2003, Abdilla *et al.*, 2007).

Oxidative stress has been associated with hypertension via several mechanisms. These include both structural (Baas and Berk, 1995, Rojas *et al.*, 2006, Papaharalambus and Griendling, 2007) and functional changes in the vasculature (Cai and Harrison, 2000) (Figure 1.4). Oxidative stress mediates structural changes through stimulation of growth signaling cascades in the vascular endothelial or smooth muscle cells (Rojas *et al.*, 2006, Papaharalambus and Griendling, 2007), which promote vascular cell proliferation, hypertrophy and collagen deposition leading to the thickening of vascular media, narrowing of vascular lumen and consequently increasing resistance to blood flow. In SHR, a model of essential hypertension (Dickhout and Lee, 1998), evidence of pre-glomerular structural changes and greater renal vascular resistance has been reported as early as 4 weeks of age, before the rise in arterial pressure (Smeda *et al.*, 1988, Christiansen *et al.*, 2002). Renal vascular resistance was demonstrated to begin to increase from 4 weeks of age and to remain elevated in older age groups of SHR when compared to age-matched WKY rats (Christiansen *et al.*, 2002). In addition, 6 weeks old SHR showed an increase in resistance in pre-glomerular vessels and efferent arterioles and a reduction in renal blood flow with a concomitant rise in blood pressure (Dilley *et al.*, 1984).

ROS mediates the functional alteration of blood vessels by inactivating NO, thereby promoting vasoconstriction (Cai and Harrison, 2000) (Figure 1.5). Under physiological conditions, NO is released from endothelial cells and causes dilatation of blood vessels by increasing intracellular cyclic guanosine monophosphate (cGMP) concentration, which then inhibits calcium influx and mobilization from intracellular stores, thereby facilitating vascular smooth muscle relaxation. However, in oxidative

stress, $O_2^{\bullet-}$ may interact with NO resulting in reduced NO bioavailability. Studies seem to suggest that hypertensive rats have reduced endothelial dependent vasodilation in several vascular beds including kidney, that has been ascribed in part to decrease NO activity (Mayhan, 1990, Ito *et al.*, 1992). Cultured endothelial cells from the aorta of 5 weeks old SHR had significantly lower NO release with an increase in $O_2^{\bullet-}$ production (Grunfeld *et al.*, 1995). Similarly, both cultured VSMC and endothelial cells from SHR aorta had an apparent decrease in NO release (Malinski *et al.*, 1993). Administration of tempol lowered blood pressure and increased NO bioavailability in SHR (Schnackenberg *et al.*, 1998). In addition to decreased levels of NO, reaction between $O_2^{\bullet-}$ and NO may lead to the production of peroxynitrite, an oxidant species, which further potentiates tissue injury (Halliwell, 1997b). Peroxynitrite converts tyrosine residues to nitrotyrosine, which is also a foot print of NO interaction with $O_2^{\bullet-}$.

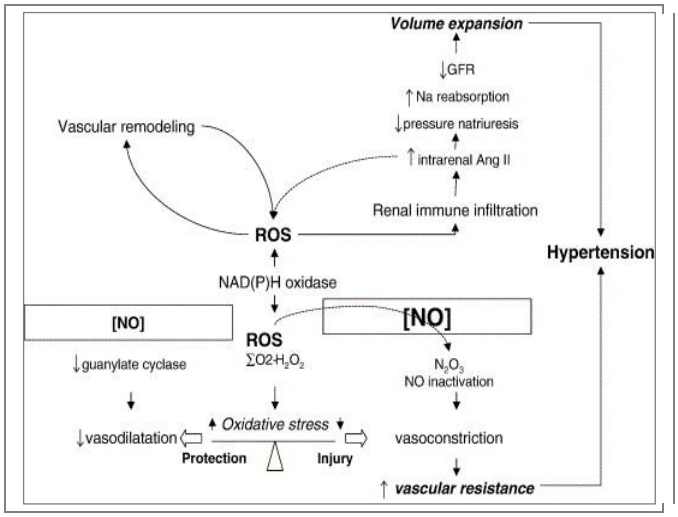


Figure 1.4 Systemic and renal pro-hypertensive role of ROS and vascular reactivity (adapted from Rodriguez-Iturbe *et al.*, 2004b).