

**AGE-RELATED CHANGES IN THE
OXIDATIVE STATUS AND ANTIOXIDANT
CAPACITY IN DIFFERENT BRAIN REGIONS
OF SPONTANEOUSLY HYPERTENSIVE RAT**

by

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**Thesis submitted in fulfillment of the
requirement for the degree
of Master of Science**

December 2005

ACKNOWLEDGEMENTS

I am most grateful to my supervisor Dr. K.N.S. Sirajudeen, Department of Chemical Pathology, for engaging me into this project and patiently teaching me free radicals and antioxidants in general. Without his encouragement and guidance this thesis would not have been completed. I am also indebted to my co-supervisor Associate Professor Nor Akmal Wahab, Department of Chemical Pathology, for critically reviewing this thesis and for his valuable comments. I would also like to thank Associate Professor Dr. H.A. Nadiger for his valuable suggestions in this project.

I wish to express my sincere gratitude to the Heads of the Department of Chemical Pathology, Department of Physiology and Animal House for providing excellent research facilities.

I deeply appreciate Mr. Chandran Govindasamy for teaching me laboratory work. I would also like to thank Dr. M. Swamy, School of Chemical Pathology, for his enormous role in the dissection of the rat brains. I am also grateful to Dr. Mohd. Ayub Saddiq, School of Dental Sciences, for his guidance and critical suggestions in statistical analysis. The technical assistance from Mr. Cheng Yew Chean in blood pressure measurement is also acknowledged.

I wish to thank all my friends for keeping my feet on the ground and bringing some perspective into my little world.

My parents, sister and brother deserve the warmest thanks for their love, patience and understanding throughout the study period.

This work was supported by Fundamental Research Grant Scheme (FRGS) [203/PPSP/617001] from Universiti Sains Malaysia, which I gratefully acknowledge.

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LIST OF ABBREVIATIONS USED

| | |
|--------------------|-----------------------------------|
| 4-HNE | 4-hydroxynonenal |
| 5-hydroxy-dC | 5-hydroxydeoxycytidine |
| 5-hydroxymethyl-dU | 5-hydroxymethyldeoxyuridine |
| 8-OHdG | 8-hydroxy-2-deoxyguanosine |
| 8-oxo-dA | 8-oxo-7,8-dihydrodeoxyadenosine |
| 8-oxo-dG | 8-oxo-7,8-dihydrodeoxyguanosine |
| A | adenine |
| ACE | angiotensin-converting enzyme |
| ACh | acetylcholine |
| AChE | acetylcholinesterase |
| AChE-E | erythrocytic acetylcholinesterase |
| AChE-R | readthrough acetylcholinesterase |
| AChE-S | synaptic acetylcholinesterase |
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| BS | brain stem |
| C | cytosine |
| CAT | catalase |
| CB | cerebellum |
| CC | cerebral cortex |
| cGPx | cytosolic glutathione peroxidase |
| Cu/Zn-SOD | copper/zinc superoxide dismutase |
| DBP | diastolic blood pressure |

| | |
|---|---|
| EC-SOD | extracellular copper/zinc superoxide dismutase |
| eNOS | endothelial nitric oxide synthase |
| G | guanine |
| GCS | glutamylcysteine synthetase |
| GI-GPx | gastrointestinal form of glutathione peroxidase |
| GPx | glutathione peroxidase |
| GR | glutathione reductase |
| GSH | reduced glutathione |
| GSSG | oxidized glutathione |
| GST | glutathione S-transferase |
| iNOS | inducible nitric oxide synthase |
| MAO | monoamine oxidase |
| MAP | mean arterial pressure |
| MDA | malondialdehyde |
| Mn-SOD | manganese superoxide dismutase |
| MPO | myeloperoxidase |
| NADH | nicotinamide adenine dinucleotide |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| Na ⁺ ,K ⁺ -ATPase | sodium-potassium adenosine triphosphatase |
| NOS | nitric oxide synthase |
| nNOS | neuronal nitric oxide synthase |
| OSI | organo-somatic index |
| PCO | protein carbonyl |
| pGPx | plasma form of glutathione peroxidase |
| PHGPx | phospholipid hydroperoxide glutathione peroxidase |

| | |
|--------|--|
| PUFA | polyunsaturated fatty acids |
| ROS | reactive oxygen species |
| RNS | reactive nitrogen species |
| SBP | systolic blood pressure |
| SHR | spontaneously hypertensive rats |
| SHR-SP | stroke-prone spontaneously hypertensive rats |
| SOD | superoxide dismutase |
| T | thymine |
| TAS | total antioxidant status |
| TBARS | thiobarbituric acid reactive substances |
| WKY | Wistar-Kyoto rats |
| XO | xanthine oxidase |

ABSTRACT

Oxidant/antioxidant imbalance has been implicated in the pathogenesis of neurological disorders associated both with aging and hypertension. Therefore, we determined oxidative status and antioxidant capacity in a time-course manner in the cerebral cortex (CC), cerebellum (CB) and brain stem (BS) of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY).

Six animals from WKY and SHR strains were sacrificed at 8, 16, 24, 32, 40, 48, 56 and 64 weeks of age after measuring their blood pressure and body weight. CC, CB and BS were dissected out, homogenized and used for the following estimations: thiobarbituric acid reactive substances (TBARS), protein carbonyl (PCO), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), reduced glutathione (GSH), oxidized glutathione (GSSG), total antioxidant status (TAS) and membrane-bound enzymes activities (Na^+,K^+ -ATPase, acetylcholinesterase - AChE).

SHR showed higher blood pressure and lower body weights at all time points studied. When compared to control, TBARS from week 24 and PCO from week 32 onwards increased significantly in all brain regions of SHR. GSH content and GSH/GSSG ratio were lower in SHR from weeks 16 and 24 onwards respectively in all brain regions. TAS and activities of SOD and GST were significantly decreased in all brain regions from 24 weeks onwards in SHR. GPx activity showed significant decrease in CB and BS from week 24 and CC from week 56 onwards in SHR. CAT activity was significantly lower in CB from week 32 and CC from week 56 onwards in SHR. There was no difference in CAT activity in BS at all time points studied. GR activity showed significant decrease in CC, CB and BS from weeks 48, 16 and 24

onwards respectively in SHR. Na⁺,K⁺-ATPase showed significant decrease in its activity from week 32 onwards in all brain regions of SHR. AChE activity was significantly lower in CC, CB and BS from weeks 24, 32 and 48 onwards respectively in SHR. All three brain regions had similar SOD activity. BS of WKY and SHR had significantly higher TAS, activities of CAT and GPx, and lower TBARS and PCO levels in comparison to CC. Similar PCO levels and GPx activity were found in CB and BS, but significantly higher TAS and CAT activity, and lower TBARS levels were found in BS compared to CB. However, GSH contents, GSH/GSSG ratio and activities of GST and GR were significantly lower in BS compared to CC and CB. CC and CB had similar TBARS and PCO levels, GSH contents and TAS, but activities of GPx, CAT and GR were significantly lower in CC compared to CB.

It is suggested that the brain regions toward oxidative stress is in the order: CC>CB>BS. Along with progression of hypertension, there is increased oxidants level and decreased antioxidants capacity with alteration in membrane-bound enzymes activities in CC, CB and BS of SHR. Thus, oxidative stress may play a role in hypertension-associated neurological diseases.

ABSTRAK

Ketidakseimbangan oksidan/antioksidan dikatakan terlibat dalam patogenesis gangguan neurologi berkaitan dengan penuaan dan hipertensi. Oleh itu, kami menentukan status oksidatif dan keupayaan antioksidan mengikut perubahan umur pada korteks serebrum (CC), serebelum (CB) dan pangkal otak (BS) tikus hipertensi spontan (SHR) dan tikus Wistar-Kyoto (WKY).

Enam ekor tikus daripada strain WKY and SHR dikorbankan pada minggu 8, 16, 24, 32, 40, 48, 56 dan 64 setelah mengukur tekanan darah dan berat badan. CC, CB dan BS dikeluarkan dengan cara diseksi, dihomogenkan dan digunakan untuk penentuan berikut: bahan reaktif asid tiobarbiturik (TBARS), karbonil protein (PCO), superoksida dismutase (SOD), katalase (CAT), glutathion peroksidase (GPx), glutathion reduktase (GR), glutathion S-transferase (GST), glutathion terturun (GSH), glutathion teroksida (GSSG), status antioksidan keseluruhan (TAS) dan aktiviti enzim terikat pada membran (Na^+, K^+ -ATPase, asetilkolinesterase – AChE).

SHR menunjukkan tekanan darah yang tinggi dan berat badan yang rendah pada semua titik masa kajian. Apabila dibandingkan dengan tikus kawalan (WKY), TBARS daripada minggu 24 dan PCO daripada minggu 32 ke atas meningkat secara signifikan pada semua bahagian otak SHR. Kandungan GSH dan nisbah GSH/GSSG adalah rendah bagi SHR daripada minggu 16 dan 24 ke atas, masing-masing pada semua bahagian otak. TAS dan aktiviti SOD dan GST menyusut secara signifikan pada semua bahagian otak daripada minggu 24 ke atas bagi SHR. Aktiviti GPx bagi SHR menunjukkan penyusutan signifikan pada CB dan BS daripada minggu 24 ke atas dan pada CC daripada minggu 56 ke atas. Aktiviti CAT bagi SHR adalah rendah secara signifikan pada CB daripada minggu 32 ke atas dan CC daripada minggu 56 ke

atas. Tidak terdapat perbezaan pada aktiviti CAT pada BS pada semua titik masa kajian. Aktiviti GR bagi SHR menunjukkan penyusutan signifikan pada CC, CB dan BS masing-masingnya daripada minggu 48, 16 dan 24 ke atas. Na^+, K^+ -ATPase menunjukkan penyusutan signifikan dalam aktiviti daripada minggu 32 ke atas pada semua bahagian otak SHR. Aktiviti AChE bagi SHR adalah rendah secara signifikan pada CC, CB dan BS masing-masingnya daripada minggu 24, 32 dan 48 ke atas. Semua tiga bahagian otak mempunyai persamaan dalam aktiviti SOD. Berbanding dengan CC, BS WKY dan SHR mempunyai TAS, aktiviti CAT dan GPx yang tinggi, dan aras TBARS dan PCO yang rendah. Terdapat persamaan dalam aras PCO dan aktiviti GPx pada CB dan BS, tetapi TAS dan aktiviti CAT yang tinggi dan aras TBARS yang rendah terdapat pada BS berbanding dengan CB. Walau bagaimanapun, kandungan GSH, nisbah GSH/GSSG dan aktiviti GST and GR adalah rendah secara signifikan pada BS berbanding dengan CC dan CB. CC dan CB mempunyai persamaan dalam aras TBARS dan PCO, kandungan GSH dan TAS, tetapi aktiviti GPx, CAT dan GR adalah rendah secara signifikan pada CC berbanding dengan CB.

Dengan ini dicadangkan bahawa kecenderungan bahagian otak terhadap stres oksidatif adalah dalam turutan: CC>CB>BS. Bersama dengan perkembangan hipertensi, terdapat peningkatan aras oksidan dan penyusutan keupayaan antioksidan berserta dengan perubahan dalam aktiviti enzim-enzim terikat pada membran pada CC, CB dan BS SHR. Oleh yang demikian, stres oksidatif mungkin memainkan peranan dalam penyakit neurologi berhubung dengan hipertensi.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Cardiovascular disease is a major public health problem in Malaysia due to its high prevalence. This disease has emerged as the principal cause of mortality in our population and hypertension is considered as a prevalent risk factor (Lim *et al.*, 2004). Hypertension is defined as systolic blood pressure (SBP) of 140 mmHg or greater and/or diastolic blood pressure (DBP) of 90 mmHg or greater or current treatment for hypertension with medication (Burt *et al.*, 1995). A survey of 17,392 individuals aged 30 and above during the National Health and Morbidity Survey 2 in 1996 showed a high prevalence of elevated blood pressure (Ministry of Health, Malaysia, 1996). The overall prevalence of hypertension among Malaysian adults was 29.9 %, with self-reported hypertension 14.0 % and undiagnosed hypertension 15.9 %. It was found that 41% of hypertensive patients had never been on medication and presented with life-threatening complications (Ministry of Health, Malaysia, 1996).

Prolonged uncontrolled hypertension is known to cause brain damage from hypertensive encephalopathy (Ryan and Irawan, 2004; Koop, 2005), stroke (Reid, 1994) and vascular dementia (Skoog *et al.*, 1996). Hypertension is also a risk factor for myocardial infarction (Whelton, 1994), congestive heart failure (Fiebach *et al.*, 1989), end-stage renal disease (Kimura *et al.*, 1996) and peripheral vascular disease (Stamler *et al.*, 1993). The question of whether elevated blood pressure alone constitutes a risk factor for development of complications in the brain among hypertensive subjects is still unclear. Free radical has been proposed as an important predisposing pathogenic mechanism in the progression of hypertension and also the

development of its complications (Ohtsuki *et al.*, 1995; Wen *et al.*, 1996; Lerman *et al.*, 2001). Several reports have documented that hypertension is associated with increased free radical production as well as reduction of antioxidant capacity (Nakazono, 1991; Tse *et al.*, 1994; Jun *et al.*, 1996; Koska *et al.*, 1999). Therefore, it is possible that increased free radical production and reduction of antioxidant capacity in hypertension have a role in the pathogenesis of hypertensive brain damage. However, at what point in the development of hypertension, increased oxidative process and/or decrease in antioxidant capacity takes place in the brain is unknown.

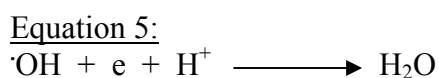
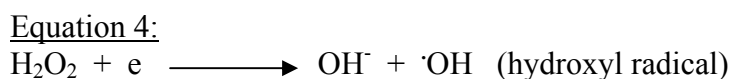
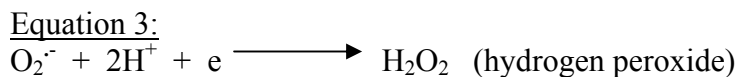
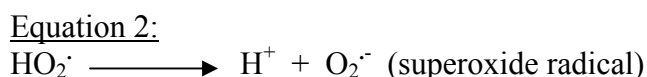
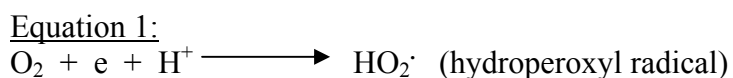
Since free radicals are produced even in normal cellular metabolism in the brain, increased production of free radicals in pathophysiological conditions exceeds the capacity of the cell to provide protection against their damaging effect, leading to oxidative stress. Thus, the balance between free radicals generation and the antioxidant defense system is crucial in determining the extent of the damage caused by these highly reactive molecules. But, there is a lack of systematic biochemical data concerning free radicals production and antioxidant defenses in the development and progression of hypertension in the different brain regions. Therefore, this study was undertaken to obtain fundamental data on oxidative status and antioxidant capacity in a time-course manner in the cerebral cortex (CC), cerebellum (CB) and brain stem (BS) of spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY), from the age of 8 weeks to 64 weeks. It is also hoped that this study will serve as a basis for future study on human hypertension.

1.2 Free radicals

A free radical can be defined as any molecular species capable of independent existence that possesses one or more unpaired electrons in its outer orbital (Gutteridge, 1995; Markesbery and Carney, 1999; Fang *et al.*, 2002). They are generally unstable and very reactive. Once radicals are formed they can either react with another radical or with another non-radical molecule by various interactions. If two radicals meet, they can combine their unpaired electron, thus forming a covalent bond. A radical has potential to generate another radical leading to the chain reaction.

Free radicals and their metabolites, reactive oxygen species (ROS) are constantly formed in the body by several mechanisms, involving both endogenous and environmental factors (Young and Woodside, 2001). Major sources of free radicals in the body include mitochondrial leak, respiratory burst, enzyme reactions, autooxidation reactions, pollutants, UV light, ionizing radiation, xenobiotics etc. ROS is a collective term that includes all reactive forms of oxygen including both the radical and nonradical species that participate in the initiation and/or propagation of radical chain reactions (Cui *et al.*, 2004). Examples of ROS which are free radicals include superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$), peroxy radical (RO_2^{\cdot}), alkoxy radical (RO^{\cdot}), hydroperoxy radical (HO_2^{\cdot}) and nitric oxide (NO^{\cdot}) (Fang *et al.*, 2002). Other ROS such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid ($HOCl$) and singlet oxygen (1O_2) are not free radicals *per se* but have oxidizing effects that contribute to oxidative stress (Cai and Harrison, 2000; Cui *et al.*, 2004).

ROS are formed in the reduction of molecular oxygen (O_2) to water as follows (Equations 1 to 5) (Gutteridge, 1995):



1.2.1 Superoxide radical ($\text{O}_2^{\cdot-}$)

$\text{O}_2^{\cdot-}$ is an anionic radical formed by the reduction of O_2 through the acceptance of a single electron (Cui *et al.*, 2004). The hydroperoxyl radical ($\text{HO}_2\cdot$), the protonated form of $\text{O}_2^{\cdot-}$, is both a more powerful oxidant and reductant than $\text{O}_2^{\cdot-}$, but $\text{HO}_2\cdot$ is unstable at physiological pH 7.4 and dissociates to $\text{O}_2^{\cdot-}$ (Gutteridge, 1995). $\text{O}_2^{\cdot-}$ has different properties depending on its solution environment. In aqueous solution $\text{O}_2^{\cdot-}$ is a weak oxidizing agent able to oxidize molecules such as ascorbic acid and thiols (Gutteridge, 1995). But $\text{O}_2^{\cdot-}$ is a much stronger reducing agent which can reduce several iron complexes such as cytochrome c and ferric-EDTA (Gutteridge, 1995). $\text{O}_2^{\cdot-}$ has limited reactivity with some proteins but is not reactive with lipids or DNA (Markesbery and Carney, 1999). $\text{O}_2^{\cdot-}$ is not membrane permeable and therefore its reaction is limited to the compartment in which it is generated (McIntyre *et al.*, 1999).

$\text{O}_2^{\cdot-}$ is mainly formed *in vivo* by the electron transport chains in the mitochondria and microsomes through electron leakage, a phenomenon that increases with an increase in O_2 utilization (Cui *et al.*, 2004). $\text{O}_2^{\cdot-}$ is also formed by metal ion-dependent oxidation of epinephrine and norepinephrine, and by the action of enzymes

such as tryptophane hydroxylase, indoleamine dioxygenase and xanthine oxygenase (Cui *et al.*, 2004). Another source of $O_2^{\cdot-}$ is cyclooxygenase which is present in cerebral extracellular space (Kontos *et al.*, 1985). It was found that $O_2^{\cdot-}$ can also be produced by brain nitric oxide synthase (NOS) from one-electron reduction of O_2 (Pou *et al.*, 1992). In addition, $O_2^{\cdot-}$ can also be generated from O_2 through nicotinamide adenine dinucleotide phosphate (NADPH) oxidation by NADPH oxidase, oxidation of xanthine or hypoxanthine by xanthine oxidase and one-electron reduction of O_2 by cytochrome P450 (Fang *et al.*, 2002).

$O_2^{\cdot-}$ will not normally react with nitric oxide (NO^{\cdot}) to yield peroxynitrite ($ONOO^{\cdot}$) and peroxynitrous acid ($HOONO$) except when NO^{\cdot} is produced in large amount. $O_2^{\cdot-}$ is not a damaging ROS if compared to its derivatives such as $\cdot OH$. It is considered biologically significant because it becomes the main source for the production of H_2O_2 and precursors for the generation of $\cdot OH$.

Equation 6:

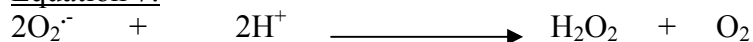


$O_2^{\cdot-}$ disappears in aqueous solution rapidly through dismutation reaction in which hydrogen peroxide and oxygen are formed (Equation 6). The reaction is greatly accelerated by the superoxide dismutase (McCord and Fridovich, 1969).

1.2.2 Hydrogen peroxide (H_2O_2)

Any biological system producing $O_2^{\cdot-}$ will also produce H_2O_2 as a result of the dismutation reaction (Gutteridge, 1995). H_2O_2 is not a free radical because it contains no unpaired electron in the outer orbital. H_2O_2 is formed by addition of an electron and $2H^+$ to the $O_2^{\cdot-}$ under the influence of superoxide dismutase. Two $O_2^{\cdot-}$ molecules can react with H^+ to form H_2O_2 and O_2 (Equation 7).

Equation 7:



This reaction is called dismutation reaction because radical reactant react together to form nonradical products (Fouad, 2003). In addition, several enzymes such as L-amino acid oxidase, glycolate oxidase, monoamine oxidase and nitric oxide synthase produces H_2O_2 directly by the transfer of two electrons to O_2 (Halliwell, 1992; Heinzl *et al.*, 1992).

Unlike the charged $\text{O}_2^{\cdot-}$, H_2O_2 crosses cell membrane freely (Halliwell and Gutteridge, 1985). This is because H_2O_2 readily mixes with H_2O and is treated as a H_2O molecule by the body and diffuses across cell membrane. Therefore H_2O_2 found in one location might diffuse to another location and cause damage to it. Damage occurs when H_2O_2 comes into contact reduced form of certain transition metals such as Fe^{2+} or Cu^+ . In the presence of transition metals, H_2O_2 is decomposed to yield the highly reactive hydroxyl radicals via the Haber-Weiss or Fenton reactions (Cui *et al.*, 2004). As H_2O_2 is lipid soluble, it can cause damage to localized Fe^{2+} containing membranes far from its site of origin (Marks *et al.*, 1996). H_2O_2 is involved in the formation of HOCl and $^1\text{O}_2$ in the presence of myeloperoxidase (MPO) from neutrophils during the destruction of foreign organisms in a response called respiratory burst (Tatsuzawa *et al.*, 1999).

1.2.3 Hydroxyl radical ($\cdot\text{OH}$)

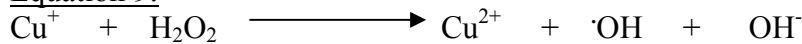
The $\cdot\text{OH}$ is an extremely aggressive oxidant that can react at great speed with almost every biological molecule found in living cells including lipid, proteins, nucleic acids and carbohydrates (Halliwell and Gutteridge, 1985). Because of its low half-life 10^{-9} s at 37°C , the direct action of $\cdot\text{OH}$ is confined to regions immediately in the vicinity of

its formation (Sies, 1993; Cui *et al.*, 2004). $\cdot\text{OH}$ can be produced experimentally by various procedures including radiation or by decomposition of peroxyxynitrite (Cui *et al.*, 2004). Although $\cdot\text{OH}$ formation can occur in various ways, the most important mechanism *in vivo* is likely to be the transition metal catalyzed decomposition of $\text{O}_2^{\cdot-}$ and H_2O_2 (Young and Woodside, 2001). H_2O_2 can react with transition metals such as iron II (Fe^{2+}) or copper I (Cu^+) in a reaction termed Fenton reaction as shown in equations 8 and 9 (Young and Woodside, 2001).

Equation 8:

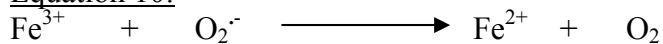


Equation 9:

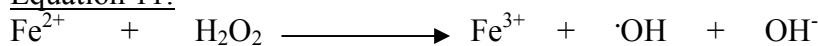


$\cdot\text{OH}$ can also be produced when $\text{O}_2^{\cdot-}$ and H_2O_2 react together directly in the iron-catalyzed reaction termed Haber-Weiss reaction (Equations 10 to 12) (Young and Woodside, 2001). But the rate constant for this reaction in aqueous solution is virtually zero.

Equation 10:

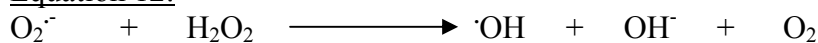


Equation 11:



net result:

Equation 12:



The net result of the above reaction is known as the Haber-Weiss reaction. Under normal circumstances, most of the iron in the body are tightly bound to one of several proteins including transferrin, lactoferrin, haem proteins, ferritin or haemosiderin. However, in pathological conditions such as active inflammation and ischaemia reperfusion injury, excessive iron may be released from its sequestered

form leading to the generation of $\cdot\text{OH}$ by Fenton or Haber-Weiss reaction (Young and Woodside, 2001).

1.2.4 Singlet oxygen ($^1\text{O}_2$)

$^1\text{O}_2$ is not a free radical because it does not have an unpaired electron. It is considered as one of ROS due to its strong oxidizing capability in which the spin restriction of two unpaired electrons with parallel spins is removed (Gutteridge, 1995). It can induce various genotoxic, carcinogenic and mutagenic effects through its action on polyunsaturated fatty acids and nucleic acid (Cui *et al.*, 2004). Formation of $^1\text{O}_2$ is extremely important in photochemical reactions. $^1\text{O}_2$ is produced in the presence of molecular oxygen in chlorophylls, retinal and flavins during pigment reaction (Fouad, 2003). $^1\text{O}_2$ can be formed *in vivo* by enzymatic activation of O_2 through lipoxygenase activity during prostaglandin biosynthesis (Cadenas and Sies, 1984). It can also be produced by physicochemical reactions such as thermal decomposition of endoperoxides and dioxetanes, reaction of ozone with human body fluids and reaction of H_2O_2 with HOCl (Cui *et al.*, 2004).

1.2.5 Nitride oxide ($\text{NO}\cdot$)

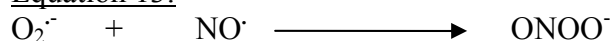
$\text{NO}\cdot$ is considered as a free radical with limited reactivity but it can react with O_2 , $\text{O}_2^{\cdot-}$ and transition metals to form more powerful oxidant (Markesbery and Carney, 1999). $\text{NO}\cdot$ is endogenously produced and initially characterized as endothelial-derived relaxing factor (Furchgott and Zawadzki, 1980). $\text{NO}\cdot$ is now found to be involved in biological actions ranging from vasodilation, neurotransmission, inhibition of platelet adherence and aggregation and macrophage and neutrophil-mediated killing of

pathogens (Moncada *et al.*, 1991). It is synthesized from L-arginine in a variety of cells and tissues by nitric oxide synthase (NOS) (Marletta, 1993; Fang *et al.*, 2002). Three isoforms of NOS account for NO[•] production including neuronal NOS (nNOS; type I) which originally identified as constitutive in neuronal tissue, inducible NOS (iNOS; type II) which is originally identified as being inducible by cytokines in activated macrophages and liver, and endothelial NOS (eNOS; type III) which is originally identified as constitutive in vascular endothelial cells (Fang *et al.*, 2002). Production of NO[•] in the central nervous system by nNOS accounts for most of NO[•] activity (Yun *et al.*, 1996). NO[•] is produced excessively in excitotoxicity, inflammation and ischaemia-reperfusion injury (Bredt and Snyder, 1994). High concentrations of NO[•] are toxic and interact with O₂^{•-} to form peroxynitrite (Beckman *et al.*, 1990).

1.2.6 Peroxynitrite (ONOO⁻)

ONOO⁻ is formed *in vivo* by the reaction of NO[•] with O₂^{•-} as shown in Equation 13 (Althaus *et al.*, 1994; Beckman and Koppenol, 1996).

Equation 13:



Formation of ONOO⁻ reduces the concentrations and biological effects of both O₂^{•-} and NO[•] in the body. But ONOO⁻ is considered as a more potent oxidant as compared to O₂^{•-} and NO[•] because it has strong oxidizing activity with membrane lipids, carbohydrates, proteins and DNA (Pryor and Squadrito, 1995). At physiological pH, ONOO⁻ is protonated to form peroxynitrous acid (HOONO), a relatively long-lived oxidant (Gutteridge, 1995). HOONO decomposes spontaneously to [•]OH and NO₂ which are potent activators of lipid peroxidation (Beckman *et al.*,

1990). ONOO^- also serves as a nitrating agent promoting the addition of nitrogroups to aromatic and indolic groups in proteins containing tyrosine, phenylalanine and tryptophan thus inactivating proteins (Markesbery and Carney, 1999).

1.2.7 Hypochlorous acid (HOCl)

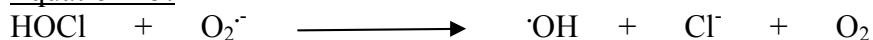
HOCl which is a powerful oxidant is formed in the body by the activated neutrophils during respiratory burst to kill organisms (Gutteridge, 1995). The heme-containing enzyme MPO present in the phagocyte cytoplasm can catalyze the formation of HOCl from H_2O_2 and chloride ions (Cl^-) (Equation 14).

Equation 14:

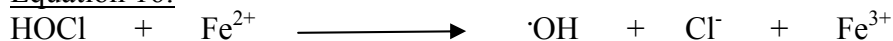


In addition, HOCl may also give rise to $\cdot\text{OH}$ by an iron-independent reaction (Equation 15) (Candeias *et al.*, 1993) and iron-dependent reaction (Equation 16) (Candeias *et al.*, 1994).

Equation 15:



Equation 16:



1.3 Cellular sources of free radicals

Oxygen is required for the generation of all ROS, reactive nitrogen species (RNS) and other reactive species. The major reactions for the production of oxygen and nitrogen free radicals in the body are illustrated in Figure 1.1 (Fang *et al.*, 2002).

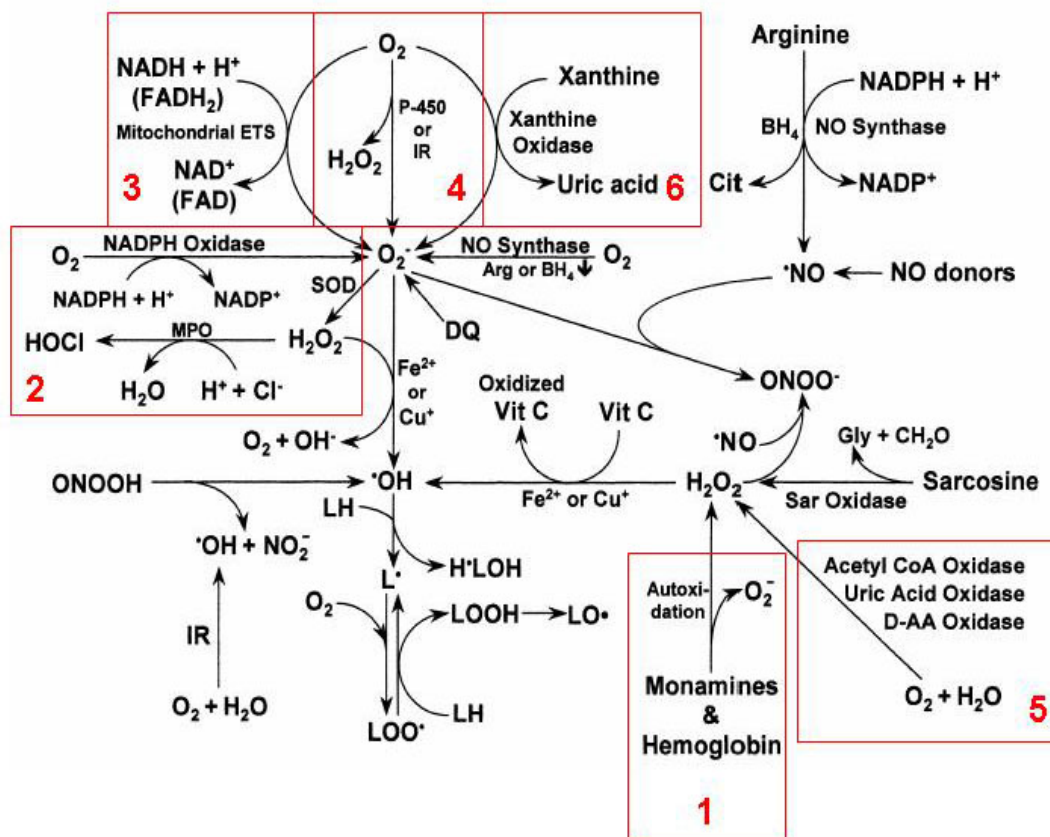


Figure 1.1: Production of oxygen and nitrogen free radicals and other reactive species in mammalian cells. AA, amino acid; Arg, L-arginine; BH_4 , (6R)-5,6,7,8,-tetrahydro-L-biopterin; CH_2O , formaldehyde; Cit, L-citrulline; DQ, diquat; ETS, electron transport system; FAD, flavin adenine dinucleotide (oxidized); $FADH_2$, flavin adenine dinucleotide (reduced); Gly, glycine; H_2O_2 , hydrogen peroxide; HOCl, hypochlorous acid; $H\cdot LOH$, hydroxyl lipid radical; IR, ionizing radiation; L \cdot , lipid radical; LH, lipid (unsaturated fatty acid); $LO\cdot$, lipid alkoxy radical; $LOO\cdot$, lipid peroxy radical; LOOH, lipid hydroperoxide; MPO, myeloperoxidase; NAD^+ , nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized); NADPH, nicotinamide adenine dinucleotide phosphate (reduced); NO, nitric oxide; O_2^- , superoxide anion radical; $\cdot OH$, hydroxyl radical; $ONOO^-$, peroxynitrite; P-450, cytochrome P-450; PDG, phosphate-dependent glutaminase; Sar, Sarcosine; SOD, superoxide dismutase; Vit C, vitamin C; Vit E, vitamin E (α -tocopherol) (Fang *et al.*, 2002).

1.3.1 Autoxidation

Autoxidation is a side reaction of the aerobic internal milieu. Biological molecules that can undergo autoxidation include catecholamines, haemoglobin, myoglobin, reduced cytochrome C, thiol, flavins, ferredoxin and cyclooxygenase (Del Maestro, 1980; Kontos *et al.*, 1985). Autoxidation of any of the above molecules in a reaction results in the reduction of the O_2 and the formation of ROS. O_2^- is the primary radical formed (Del Maestro, 1980) (Figure 1.1-1).

1.3.2 Respiratory burst

Respiratory burst is an antimicrobial defense system. It aims to damage the membranes, DNA and other cellular components of invading organism. Activated macrophages, neutrophils, monocytes and eosinophils produce O_2^- and H_2O_2 as one of the mechanisms to kill bacteria and fungi and to inactivate viruses (Halliwell, 1997). It is also a potentially dangerous mechanism if it is activated inappropriately. This is exactly what happens in people with chronic inflammatory diseases such as inflammatory bowel disease or rheumatoid arthritis (Halliwell, 1997). Activation of cell membrane enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase by immunoglobulin-coated bacteria, immune complexes, complement 5a or leukotriene will initiate the respiratory burst with generation of O_2^- (Fouad, 2003). HOCl generated by the action of MPO is bactericidal that attacks the membranes of bacteria and subsequently lysis of bacteria occurs (Figure 1.1-2).

1.3.3 Mitochondrial leak

Free radicals are constantly formed in the cells by normal metabolic processes, as the reduction of O_2 to H_2O by the mitochondrial electron transport chain (Figure 1.1-3). The main sites of $O_2^{\cdot-}$ production are in the mitochondrial respiratory chain of enzymes which includes nicotinamide adenine dinucleotide dehydrogenase (complex I) and ubiquinone Q-cytochrome b complex (complex III) (Boveris and Chance, 1973; Chance *et al.*, 1979; McIntyre *et al.*, 1999). Production of $O_2^{\cdot-}$ is regulated by the respiratory chain carriers which includes NAD-linked substrates, succinate, adenosine diphosphate (ADP) and O_2 (Boveris and Chance, 1973). In the mitochondrial respiration, O_2 itself is reduced in such way that two electrons and two pairs of protons are accepted by each oxygen atom leading to the formation of a H_2O molecule. But mitochondrial electron transport system is not always perfect. There is a constant leak of a few electrons into the mitochondrial matrix, leading to univalent reduction of O_2 that forms $O_2^{\cdot-}$ (Becker *et al.*, 1999). Under physiologic conditions, about 1% to 3% of the O_2 consumed by the body is converted into $O_2^{\cdot-}$ and other ROS as by-products (Tritschler *et al.*, 1994; Sohal and Weindruch, 1996). Production of $O_2^{\cdot-}$ by the mitochondria increases when the respiratory chain is under reduced conditions or when mitochondria are damaged (Chance *et al.*, 1979). As mitochondria are the major sites of $O_2^{\cdot-}$ production, paradoxically they also become the main target of free radical attacks. ROS generated by mitochondria can cause damage to mitochondrial components and initiate degradative processes (Cadenas and Davies, 2000). Increased production of $O_2^{\cdot-}$ and H_2O_2 in the mitochondria is associated with aging (Ames *et al.*, 1995). It has been found that $O_2^{\cdot-}$ overproduced in a mitochondrial compartment when uncoupled from antioxidant defenses induces impairment of mitochondrial function (Murakami *et al.*, 1998).

1.3.4 Microsomes

The microsomal cytochrome P450 enzymes are composed of two functional units embedded in the membrane of the endoplasmic reticulum:- (1) cytochrome P450 which binds the substrate and oxygen and carries out the reaction, (2) cytochrome P450 reductase which transfers electrons from nicotinamide adenine dinucleotide phosphate (NADPH) (Marks *et al.*, 1996) (Figure 1.1-4). There are about 100 different P450 isoenzymes in the human with different but overlapping specificities (Marks *et al.*, 1996). It was shown that isolated microsomes can generate $O_2^{\cdot-}$ and H_2O_2 with nicotinamide adenine dinucleotide (NADH) and NADPH supplement (Kathan and Ullrich, 1982). The microsomal cytochrome P450 enzymes are formed abundantly in the liver in which they involve in xenobiotic metabolism. It was shown that cytochrome P450 reductase can reduce inorganic compounds such as paraquat (Brigelius and Anwer, 1981) and diquat (Smith *et al.*, 1985) to form an unstable organic radical. Then, the unstable radical donates its electron to O_2 to form $O_2^{\cdot-}$, which can undergo one-electron reduction to generate more potent reactive radicals.

1.3.5 Peroxisomes

Peroxisomes contain several enzymes which include D-amino acid oxidase and fatty acyl-CoA oxidase that generate H_2O_2 but not $O_2^{\cdot-}$ (Chance *et al.*, 1979) (Figure 1.1-5). Liver is the primary organ which contributes significantly to the overall H_2O_2 production. H_2O_2 can be generated in peroxisomes by degradation of long-chain fatty acids by fatty acyl-CoA oxidase (Conway *et al.*, 1987). Prolonged starvation or fasting can induce H_2O_2 production through peroxisomal oxidation of fatty acids (Conway *et al.*, 1987).

1.3.6 Cytosol

Xanthine oxidoreductase is responsible for formation of ROS in the cytosol. It is involved in catalyzing the oxidation of hypoxanthine and xanthine in the process of purine metabolism (Cai and Harrison, 2000). Xanthine oxidoreductase can exist in two interconvertible forms either as xanthine dehydrogenase or xanthine oxidase. The former reduces NAD^+ whereas the latter prefers O_2 , leading to the production of both O_2^- and H_2O_2 . Both enzymes can be found abundantly in sinusoidal endothelial cells, hepatocytes and Kupffer cells (Wiezorek *et al.*, 1994). In pathological conditions such as ischaemia reperfusion, production of xanthine and xanthine oxidase are greatly enhanced, thus providing xanthine oxidase with the reducing equivalents it needs to convert O_2 to O_2^- and H_2O_2 (Xia and Zweier, 1995). This is because, during reoxygenation, ATP is converted to ADP, AMP and adenosine, which can be degraded further to inosine, hypoxanthine and xanthine. Xanthine oxidase catalyzes the reaction of hypoxanthine to xanthine and subsequently oxidizes xanthine to produce uric acid, O_2^- and H_2O_2 (Parks and Granger, 1986) (Figure 1.1-6).

1.4 Oxidative stress and cellular damage

Free radicals have been previously shown to be capable of causing cellular damage to biomolecules such as lipids, proteins and nucleic acids (Sohal *et al.*, 1995; Cai *et al.*, 1996; Liu *et al.*, 1996; Cai and Harrison, 2000). Oxidative stress is a condition when the level of free radicals exceeds the endogenous antioxidant defense mechanisms of the host which can be due to an increased production of free radicals and/or a decrease of antioxidant defenses (Markesbery and Carney, 1999; Sanchez-Alvarez *et al.*, 2002). When the increased demand on the cell's capacity to detoxify free radicals is not met, free radicals oxidize biomolecules, leading to accumulation of toxic

oxidation products such as aldehydes or isoprostanes from lipid peroxidation, protein carbonyls from protein oxidation and oxidized base adducts from DNA oxidation. These oxidized products can be used as markers for excess oxidative status.

1.4.1 Lipid peroxidation

Lipids are probably the most susceptible biomolecules to free radical attack. Cell membranes are the major site of lipid peroxidation because they are rich in polyunsaturated fatty acids (PUFA). The presence of a double bond in the PUFA side chains of membrane lipids weakens the C-H bonds on the carbon atom adjacent to the double bond which allows easy removal of H⁺. Lipid peroxidation causes gradual loss of membrane fluidity and membrane potential. Membrane permeability to ions such as Ca²⁺ is increased and if continued long enough can lead to loss of membrane integrity (Halliwell and Gutteridge, 1985). Membrane-bound enzymes and receptors are also inactivated. A study has shown that modifications of lipid composition may alter Na⁺,K⁺-ATPase activity (Sanderman, 1978). Degradation of fatty acids leads to formation of aldehydes including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) which are cytotoxic to cells (Esterbauer *et al.*, 1991). MDA is produced during peroxidation of fatty acids of both the n-6 series such as, linoleic and arachidonic acids and the n-3 series such as, docosahexaenoic acid (Pryor and Stanley, 1975).

The oxidation of lipids by free radicals generally consists of three steps: initiation, propagation and termination (Halliwell and Gutteridge, 1985; Gutteridge, 1995). (1) Initiation, in which a free radical compound such as hydroxyl radical extracts a hydrogen atom from a polyunsaturated lipid (LH), resulting in the formation of a lipid radical (L[•]) (Equation 17). (2) Propagation, in which the lipid radical undergoes molecular rearrangement to form a conjugated diene which then

reacts with O₂ to give rise to a lipid peroxy radical (LOO[•]) (Equation 18). Lipid peroxy radical, in turn, starts a self-perpetuating chain reaction, abstracts a hydrogen atom from another fatty acid to form lipid hydroperoxide (LOOH) and lipid radical (L[•]) (Equation 19). LOOH formed during the chain reaction are a complex mixture of isomers. They are stable molecules at physiological temperature but their decomposition is catalyzed by transition metals. (3) Termination, in which the radicals themselves, or between the radicals and antioxidants, giving rise to nonradical products or unreactive radicals. For example, two peroxy radicals annihilate each other to terminate the chain by forming of cyclic peroxide (LOOL) (Equation 20). One peroxy radical and one lipid radical annihilate each other to form cyclic peroxide (Equation 21). This chain reaction can also be terminated by antioxidants such as vitamin E (*α*-tocopherol) by donating hydrogen atom to peroxy radical, stopping it from initiating (Equation 22). This leaves behind an unpaired electron on the vitamin E. But vitamin E radical is unreactive and degrades harmlessly. Vitamin E radical can also be reduced back to vitamin E by ascorbic acid (vitamin C) which is also an antioxidant (Slater, 1984). The peroxy radicals formed in lipid peroxidation survive long enough to be able to move to new fatty acid molecules. So they can readily be scavenged by various antioxidants.

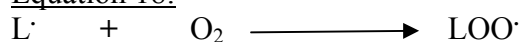
Initiation

Equation 17:

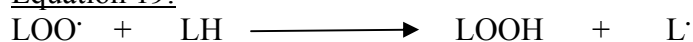


Propagation

Equation 18:

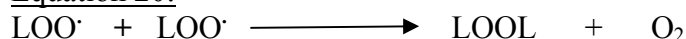


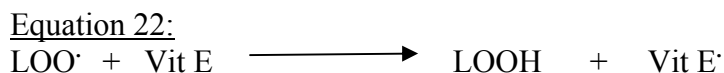
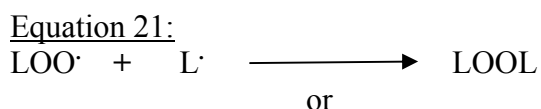
Equation 19:



Termination

Equation 20:





Lipid peroxidation has been quantitatively assessed by measuring (1) MDA levels by the thiobarbituric acid reactive substances (TBARS) assay, (2) alterations in PUFA and (3) the breakdown products of PUFA, such as aldehydes and isoprostanes (Markesbery and Carney, 1999). Normally, TBARS test is used as an indicator of the major lipid peroxidation burden because it is easy to perform and inexpensive (Moore and Roberts, 1998) even though they also measure a variety of products including non-lipid derived MDA, C₃ to C₁₀ aldehydes and species resulting from chemical interaction among non-lipid molecules during the assay (Markesbery and Carney, 1999). No single method is adequate for all stages of lipid peroxidation in a biological system (Gutteridge, 1995).

1.4.2 Protein oxidation

Proteins are less vulnerable than PUFA to free radical attack. Following exposure to free radicals, proteins can undergo certain types of modifications including the oxidation of the amino acid residues and/or peptide backbone of proteins resulting in the generation of protein carbonyl (PCO) (Marnett *et al.*, 2003). The process is initiated by hydrogen abstraction from the α -carbon in a peptide chain. If two proteins radicals are in close proximity, they may cross-link with one another by radical coupling. Alternatively, molecular oxygen can attack the α -carbon-centred radical to form peroxide intermediates leading to rearrangement and subsequent cleavage of the peptide bond to form carbonyl-containing peptides (Dean *et al.*, 1997). Carbonyl

formation is an important detectable marker of protein oxidation that can be measured by reaction of 2,4-dinitrophenylhydrazine with proteins to form the corresponding hydrazones (Evans *et al.*, 1999; Marnett *et al.*, 2003).

In proteins, the amino acids such as cysteine, tyrosine and methionine are particularly vulnerable to modifications by O_2^- , $\cdot OH$, HOCl, peroxynitrous acid (ONOOH) and nitrosoperoxycarbonate ($ONO_2CO_2^-$) (Marnett *et al.*, 2003). Reversible oxidation of the sulfhydryl group on cysteine converts it to cysteine sulfenic acid, which can react with thiols or undergo further irreversible oxidation to a sulfinic acid and a sulfonic acid (Claiborne *et al.*, 1999). A variety of oxidative modifications can occur to tyrosine side chains in proteins, including formation of *0,0'*-dityrosine, 3,4-dihydroxyphenylalanine, 3-nitrotyrosine and 3-chlorotyrosine (Marnett *et al.*, 2003). Oxidation of methionine residues in proteins results in the formation of methionine sulfoxide (Levine *et al.*, 2000). Free radicals modify proteins that play important roles in biological functions. Protein oxidation would be expected to affect a variety of cellular functions involving proteins including protein synthesis, energy production, signal transduction and transport systems (Evans *et al.*, 1999; Stadtman and Levine, 2000; Sohal, 2002; Marnett *et al.*, 2003). Protein oxidation may have secondary effect to other biomolecules such as development of new antigens provoking autoimmune responses (Evans *et al.*, 1999). Carbonyl groups may also be introduced into proteins by glycation and reaction with glycoxidation and lipid peroxidation products (Butterfield and Stadtman, 1997). Lipid peroxidation products such as MDA and 4-HNE have been shown to react with cysteine, histidine and lysine residues in proteins (Uchida, 2000). Some common protein targets for free radicals attack are shown in Table 1.1.

Table 1.1: Some common protein targets for free radicals attack
(Marnett *et al.*, 2003)

| Protein targets | Residue modified | Modifying agents | Biological consequences |
|---|--|--|--|
| <u>Enzymes</u> | | | |
| Caspases | Catalytic cysteine | HO [•] , nitrosating agents | Glutathionylation, inactivation |
| Protein tyrosine Phosphatases | Catalytic cysteine, active-site tyrosine | HO [•] , O ₂ ^{-•} , nitrosating agents, ONOOH | Glutathionylation, inactivation, accumulation of phosphotyrosine |
| Tyrosine hydroxylase | Active-site tyrosine | ONOOH | Inhibition of dopamine synthesis |
| Mn-SOD | Active-site tyrosine | ONOOH | Prevention of O ₂ ^{-•} detoxification |
| <u>Structural and membrane proteins</u> | | | |
| Tubulin | cysteine residue | 4-HNE, ONOOH | Disruption of microtubule networks |
| N-methyl-D-aspartate receptor channel | Extracellular cysteine residue | Nitrosating agents | Inactivation, reduced Ca ²⁺ influx |
| <u>Transcription factors</u> | | | |
| AP-1 | Cysteine in DNA-binding domain | Various oxidants, NO | Glutathionylation, inhibition of DNA binding |
| OxYr | Cysteine residues | HO [•] , nitrosating agents | Glutathionylation, transcriptional activation of OxYr-responsive genes |

1.4.3 DNA oxidation

Free radicals can also attack DNA molecule and cause DNA damage. Oxidative alterations of DNA molecules include strand breaks, sister chromatid exchange, DNA-DNA and DNA-protein crosslinking and base modifications (Cochrane, 1991; Davies, 1995). DNA can be damaged by hydroxyl radical, nitric oxide, halogen and lipid peroxidation products such as MDA and 4-HNE (Marnett *et al.*, 2003).

Hydroxyl radical is particularly damaging because it is capable of modifying purine and pyrimidine bases of DNA as well as sugar backbones of DNA. Hydroxyl radical can add to double bonds of DNA bases or abstract hydrogen atoms from either methyl groups or deoxyribose residues (Chatgililoglu and O'Neill, 2001). Hydroxyl radical can also add to the 7,8 double bond of purines to produce the 8-oxo-7,8-dihydrodeoxyguanosine (8-oxo-dG). Hydroxyl radical reacts with pyrimidines by adding to the 5,6 double bond to generate a carbon-centred radical that reacts with molecular oxygen to form a hydroperoxide which is then reduced to 5-hydroxymethyldeoxyuridine (5-hydroxymethyl-dU) (Marnett *et al.*, 2003). 8-oxo-dG has been shown capable of inducing transversions of guanine (G) to thymine (T) or cytosine (C) in both *in vitro* replication experiments and *in vivo* mutagenesis experiments (Tan *et al.*, 1999; Gentil *et al.*, 2000). 8-oxo-7,8-dihydrodeoxyadenosine (8-oxo-dA) has been shown to induce adenine (A)→C mutations in *in vitro* replication experiments (Shibutani *et al.*, 1993). Hydroxyl radical damage to pyrimidines produces 5-Hydroxydeoxycytidine (5-hydroxy-dC) which can induce C→T and C→A mutations *in vitro* and C→T transitions *in vivo* (Feig *et al.*, 1994). The nonspecific binding of Fe²⁺ to DNA stimulates localized production of the hydroxyl radical which can cause strand breaks and base alterations in the DNA (Marks *et al.*, 1996). Free radical attack on DNA has been studied by analyses of all

these modified bases. One of the most studied oxidatively modified nucleoside is 8-hydroxy-2'-deoxyguanosine (8-OHdG), which derives from hydroxyl attack on deoxyguanosine (Shigenaga and Ames, 1991). Some mutagenic consequences of replication of endogenous DNA adducts are shown in Table 1.2.

Table 1.2: Mutagenic consequences of replication of endogenous DNA adducts (Marnett *et al.*, 2003)

| Damage type | Mutations |
|-----------------------------|-----------|
| <u>Adenine</u> | |
| 8-Oxo-dA | A→C |
| Etheno-dA | A→G |
| <u>Cytosine</u> | |
| 5-Methyl-dC | C→T |
| Etheno-dC | C→A, C→T |
| <u>Guanine</u> | |
| O ⁶ -methy-dG | G→T |
| M ₁ dG | G→T, G→A |
| 8-Bromo-dG | G→T |
| 8-Oxo-dG | G→A |
| 1,N ² -etheno-dG | G→A, G→T |
| <u>Thymine</u> | |
| Thymine glycol | T→C |

1.5 Free radicals and membrane-bound enzymes

The cellular membrane of the brain is abundant with enzymes like sodium-potassium adenosine triphosphatase (Na^+, K^+ -ATPase) and acetylcholinesterase (AChE). Since brain membrane is rich in PUFA, the oxidation of membrane lipids may lead to inactivation of these membrane-bound enzymes.

1.5.1 Na^+, K^+ -ATPase

Na^+, K^+ -ATPase (EC 3.6.1.3) is a membrane-bound enzyme composed of two subunits: an α -catalytic subunit with a relative molecular weight of 90-110 kDa and a β -subunit with a molecular mass of 40-60 kDa (Kourie, 1998). It is a crucial enzyme responsible for maintaining the ionic gradient necessary for neural excitability. It is present at high concentrations in the brain cellular membrane, consuming about 40-50% of the ATP generated in this tissue (Erecinska and Silver, 1994). Na^+, K^+ -ATPase utilizes the energy derived from ATP hydrolysis to pump out Na^+ from inside the cell and to transfer K^+ from outside to cytosol against their concentration gradients, generating internal negative charges (Chakraborty *et al.*, 2003). This membrane-bound enzyme requires phospholipids for its activity and is highly vulnerable to oxidative stress and the mechanism of inactivation under this condition involves disruption of phospholipid microenvironment of the enzyme or direct damage to enzyme protein by reactive oxygen radicals or lipid peroxidation products (Jamme *et al.*, 1995; Fleuranceau-Morel *et al.*, 1999; Lehtosky *et al.*, 1999). The inactivation of Na^+, K^+ -ATPase leads to partial membrane depolarization, allowing excessive Ca^{2+} entry inside the neurons resulting in toxic events like excitotoxicity (Chakraborty *et al.*, 2003). It was found that Na^+, K^+ -ATPase activity is decreased in cerebral ischaemia (de Souza Wyse *et al.*, 2000) and in various neurodegenerative disorders

such as Alzheimer's disease (Lees, 1993; Hattori *et al.*, 1998). The inhibitory effects of iron-generated free radicals on the activity of Na⁺,K⁺-ATPase of red blood cells can be reversed by antioxidants (Rohn *et al.*, 1993).

1.5.2 AChE

AChE (EC 3.1.1.7) is a type B carboxylesterase that rapidly hydrolyzes the neurotransmitter acetylcholine (ACh) at brain cholinergic synapses as well as at neuromuscular junctions (Taylor and Radic, 1994). Neurotransmission mediated by ACh contributes to numerous physiological functions (Borovicka *et al.*, 1997) as well as to memory, learning and panic response (Everitt and Robbins, 1997; Battaglia, 2002). There are three AChE isoforms: synaptic (AChE-S), readthrough (AChE-R) and erythrocytic (AChE-E) (Grisaru *et al.*, 1999). AChE-S constitutes the principal multimeric enzyme in brain and muscle; AChE-R appears in embryonic and tumor cells and is induced under psychological, chemical and physical stress; and AChE-E associates with red blood cell membrane. It has been shown that various forms of AChE have experimentally identical catalytic properties (Schwarz *et al.*, 1995). The classical role of AChE is to terminate cholinergic neurotransmission by hydrolysis of ACh. However, it was found that AChE is co-released from the dopaminergic neurons, implying an interaction between AChE and dopamine which is important for the dopaminergic function (Klegeris *et al.*, 1995). There is evidence that abnormality in central cholinergic system of SHR may contribute to the development and/or maintenance of hypertension (Buccafusco and Spector, 1980; Makari *et al.*, 1989). For example, intravenous injection of AChE inhibitor evoked an enhanced hypertensive response in SHR as compared with WKY (Buccafusco and Spector, 1980; Makari *et al.*, 1989). Intracerebroventricular injection of cholinergic blocker