Inflammation

Since birth, humans and animals are continuously exposed to a stream of pathogens (viruses, bacteria, protozoans, fungi, metazoans etc.) as well as various harmful agents, which have the potential to derange homeostasis. Without effective protective mechanisms, each of us would succumb to diseases caused by harmful effects of foreign substances. In the battle against these invaders, humans and other vertebrates have evolved a complex array of defensive mechanisms; inflammation being the vital component of this host defense. Inflammation is a complex response to local injury or other trauma. The inflammatory reaction, which is phylogenetically and ontogenetically the oldest defense mechanism, includes increased blood supply to the site of damage through vasodilation (via increased permeability caused by retraction of endothelial cells), which allows the larger cells and soluble mediators of inflammation from circulation to the site of damage. Inflammation is characterised by redness, heat, swelling, pain and sometimes loss of function.

As inflammatory response develops, various cytokines and other inflammatory mediators induce the expression of endothelial cell adhesion molecules (CAMs) leaving the endothelium activated or inflammed, to which neutrophils are the first cells to bind and extravasate into the tissues. The process of neutrophil migration can be divided into four sequential steps (1) rolling, (2) activation by chemoattractants, (3) arrest & adhesion and (4) transendothelial migration. Chemoattractants are either permanently expressed on endothelial cells or are secreted locally by cells involved in inflammatory response. Chemoattractants include a large family of chemoattractive cytokines — chemokines, e.g. IL-8, a macrophage inflammatory protein (MIP-1β). Other chemoattractants include platelet-activating factor (PAF), complement split products (C5a, C3a, C5b67) and various N-formyl peptides produced by the breakdown of bacterial proteins during infection. Binding of chemoattractants with the receptors on neutrophil surface triggers the activation signal mediated by G-proteins associated with the receptor. The signal induces a conformational
change in the integrin molecules in neutrophil membrane, increasing their affinity for CAMs on endothelium. Subsequently, neutrophils migrate to the tissue by still unknown mechanisms, which are thought to be mediated by further activation by chemoattractants. Various subsets of lymphocytes exhibit directed extravasation at inflammatory sites. During an inflammatory response, different cells including mast cells, platelets, neutrophils, monocytes/macrophages, eosinophils and lymphocytes, release a variety of mediators of inflammation. These mediators serve to trigger or enhance specific aspects of inflammatory response. These mediators include chemokines, plasma enzyme mediators, lipid inflammatory mediators and cytokine inflammatory mediators.

**Chemokines** are a superfamily of small polypeptides, which specifically control adhesion, chemotaxis, and activation of many types of leukocytes. Their action is mediated by membrane bound receptors. On binding with specific chemokines, these receptors activate G-proteins that initiate signal transduction processes that generate secondary messengers as cyclic adenosine monophosphate (cAMP), Ca$^{2+}$, inositol triphosphate (IP$_3$). Within seconds of chemokines addition, leukocytes undergo abrupt and extensive changes in shape and activation of integrins and generation of oxygen radicals by phagocytes starts. Other changes include release of granular contents, proteases in neutrophils and macrophages, histamine from basophils and cytotoxic proteins from eosinophils.

**Plasma Enzyme Mediators**: plasma contains four interconnected mediator-producing systems.

- Kinin system.
- Clotting system
- Fibrinolytic system
- Complement system

When tissue damage occurs, these four systems are activated to form a web of interacting systems that generate a number of mediators of inflammation. On tissue damage, activation of Hageman factor (a plasma clotting factor) leads to the production of bradykinin, which is a potent basic peptide that increases vascular permeability, causes vasodilation, induces pain and contraction of smooth muscles. Hageman factor also leads to large quantities of thrombin,
which induces formation of fibrinopeptide-fibrin clot. The resulting clot prevents loss of blood and spread of invading pathogen. Fibrinopeptides act as inflammatory mediators that induce increased vascular permeability and neutrophil chemotaxis. The clot is removed by proteolytic enzyme plasmin, which is an end product of the fibrinolytic system. The breakdown of fibrin clots leads to degradation products that are chemotactic for neutrophils. Complement split products like C3a, C4a, C5a also serve as important mediators of inflammation by inducing degranulation, release of histamine and other pharmacologically important mediators, cause smooth muscle contraction, increase permeability, enhance adhesion of macrophages and neutrophils to endothelium and hence their extravasation.

**Lipid Inflammatory Mediators:** Membrane perturbations following tissue damage lead to degradation of phospholipids into free arachidonic acid and lysoplatelet activating factor. The latter is subsequently converted into platelet activating factor (PAF). This factor causes platelet activation and has many inflammatory effects including eosinophil chemotaxis and activation and degranulation of neutrophils and eosinophils. Metabolic products of arachidonic acid i.e. prostaglandins (PGs) and thromboxanes produced by different cells have diverse biological effects. Arachidonic acid (AA), a precursor for prostaglandin synthesis, is an essential 20-carbon polyunsaturated fatty acid, consumed in diet or derived from elongation and desaturation of linoleic acid and occurs almost exclusively at position–2 of membrane phospholipids (Wahle, 1990; Eberhart & DuBois, 1995). The first step in the synthesis of PGs is the release of free arachidonate by hydrolysis catalysed by phospholipases. The step is essential as only free arachidonic acid acts as a substrate for further metabolism catalysed by three different oxygenases: cytochrome P450, lipooxygenases and cyclooxygenases. Availability of free AA is the rate limiting determinant in PG synthesis. Cytochrome P450 converts AA to epoxyarachidonic acids (Capdevila et al., 1992); lipooxygenases introduce one molecule of O₂ to AA to produce a series of isomeric hydroperoxyacid products including leukotriens and lipoxins (Sammuelsson et al., 1987) and cyclooxygenases oxygenate arachidonic acid (AA) to prostaglandin G₂ (PGG₂).
This unstable intermediate is then converted by peroxidase activity of cyclooxygenase to PGH$_2$, which is a common precursor for all prostanoids. PGH$_2$ is further metabolised by different but specific synthases to produce a variety of eicosanoid products (Smith et al., 1991) e.g. PGs, thromboxanes, prostacyclins. PGs are synthesised rapidly on cell stimulation and secreted immediately to act locally as autacoids or hormones through cell surface G-protein linked receptors (Gusovsky, 1991). The production of specific amounts of variety of PGs and thromboxanes depend on the exact composition of different synthases in different types of cells. Each of the products has its own range of diverse physiological effects as summarised in Table 2.1, which has been derived from literature (Arita et al., 1989; Kerins et al., 1991; Soll et al., 1991; Eberhart & DuBois, 1995; Subbaramaiah et al., 1997; Campbell & Haluschka, 2001). In addition to vascular dilation, PGs also induce neutrophil chemotaxis. The thromboxanes cause platelet aggregation and constriction of blood vessels. Leukotriens (LTB$_4$, LTC$_4$, LTD$_4$, LTE$_4$) induce smooth muscle constriction. LTB$_4$ is a potent chemoattractant of neutrophils. Leukotrienes are produced by a variety of cells including monocytes, macrophages and mast cells.

**Table 2.1: Physiological functions of prostaglandins**

<table>
<thead>
<tr>
<th>Prostaglandin</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGE$_2$</td>
<td>Pyrogenicity</td>
</tr>
<tr>
<td>PGI$_2$, PGE$_2$, PGD$_2$</td>
<td>Vasodilatory, increase vascular permeability, Kidney &amp; Urine formation</td>
</tr>
<tr>
<td>TXA$_2$, PGD$_2$</td>
<td>Vasoconstrictor, Platelet aggregation</td>
</tr>
<tr>
<td>PGF$_{2a}$, PGE$_2$</td>
<td>Reproduction, ovulation, lutenisation, sperm migration, fertilisation, implantation, fetal development, Maintaining normal gastrointestinal &amp; kidney functions.</td>
</tr>
<tr>
<td>TXA$_2$</td>
<td>Vasoconstriction</td>
</tr>
<tr>
<td>LTC$_4$, LTD$_4$, LTE$_4$</td>
<td>Contraction of smooth muscle, increase in vascular permeability</td>
</tr>
<tr>
<td>LTB$_4$, 5-HETE, PAF</td>
<td>Chemotoxis of phagocytes</td>
</tr>
<tr>
<td>TXA$_3$, PAF</td>
<td>Platelet aggregation</td>
</tr>
</tbody>
</table>
**Cytokine Inflammatory Mediators:** A number of cytokines play a significant role in the development of an acute or chronic inflammatory response. IL-1, IL-6, IL-12 and chemokines exhibit redundant and pleiotropic effects and together contribute to inflammatory response. In addition IFN-γ, by acting later, contributes to chronic inflammation by attracting and activating macrophages.

**The inflammatory process**

The inflammatory process involves series of events that can be elicited by numerous stimuli e.g. infectious agents, ischemia, antigen-antibody interactions, thermal or physical injury etc. In general, an acute inflammatory response has a rapid onset and lasts a short while, which is characterised by local vasodilation and increased capillary permeability. Acute inflammation is generally accompanied by a sub-acute phase characterised by rapid alteration in the levels of several plasma proteins. Several classes of leukocytes play essential role in inflammation. Studies have examined the role of endothelial cells, cell adhesion molecules (E-selectin, P-selectin, L-selectin), intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) and leukocyte integrins in the adhesion of leukocytes and platelets on endothelium at the sites of inflammation (Gallin et al., 1992, Bevilaqua & Nelson, 1993; Cronstein & Weissman, 1993). Activated endothelial cells play a key role in ‘targeting’ circulating cells to inflammatory sites. Expression of various adhesion molecules varies among different cell types, e.g. E-selectin expression is restricted primarily to endothelial cells and is enhanced at the sites of inflammation by cytokines like IL-1, TNF-α. P-selectin is expressed predominantly on platelets and endothelial cells and is enhanced by cytokines, thrombin and histamine. L-selectin is expressed on leukocytes and acts as a receptor for P-selectin. L-selectin is shed when these cells are activated. Cell adhesion appears to occur by recognition of cell-surface glycoproteins, and carbohydrates on circulating cells by adhesion molecules on resident cells. This endothelial activation results in adhesion of leukocytes by their interaction with newly expressed L and P selectins. E-selectin interacts with sialylated Lewis–X and other glycoproteins on the leukocyte surface; endothelial ICAM-1 interacts with leukocyte integrins. On reaching to the site of damage, neutrophils
phagocytose invading pathogens and release certain inflammatory mediators, like MIP-1α, MIP-1β and chemokines that attract macrophages to the site of damage. Macrophages arrive some hours after an inflammatory process begins. These activated macrophages exhibit enhanced phagocytosis and increased release of mediators and cytokines that contribute to the inflammatory response. Activated tissue macrophages secrete IL-1, IL-6 and TNF-α, which play essential role in inflammatory process (Dinarello, 1992). All three cytokines act locally, inducing coagulation, an increase in vascular permeability and promote the expression of certain genes to induce the synthesis of variety of proteins that contribute to inflammatory events. For example, TNF-α stimulates the expression of E-selectin, IL-1 and induces increased expression of ICAM-1 and VCAM-1. IL-1 and TNF-α in turn also act on macrophages and endothelial cells to induce the production of chemokines to enhance the influx of neutrophils. IL-1 and TNF-α are principle mediators of biological responses to LPS and other infectious stimuli. IL-1 & TNF-α work in concert with growth factors granulocyte-macrophage – colony stimulating factor (GM-CSF) and other cytokines (IL-8) and many chemokines to promote neutrophil infiltration and activation. Each of the cytokines IL-1, TNF-α, IL-6 act on hypothalamus to induce a fever response. Within 12-24 hr of the onset of the acute phase of inflammatory response, increased levels of IL-1, IL-6 and TNF-α induce the production of acute phase proteins by hepatocytes. TNF-α also acts on vascular endothelial cells and macrophages to induce the secretion of colony stimulating factors (GM-CSF, G-CSF, M-CSF), which stimulate haematopoiesis resulting in transient increase in number of white blood cells, needed to fight infection. Ability of cytokines (IL-1, IL-6, TNF-α, LIF) to induce acute phase proteins by liver results from the induction of a common transcription factor, NF-IL6. NF-IL6 contains a leucine-zipper and a basic DNA binding domain.

IL-1 comprises of two distinct polypeptides (IL-1α and IL-1β) that bind to the same cell receptor and produce similar biological responses. There are two types of IL-1 receptors: 80 kDa IL-1 receptor type 1 and 68 kDa IL-1 receptor type 2, which are present in different types of cells. TNF-α, originally termed as
'cachectin' is composed of two closely related proteins: mature TNF (TNF-α) and lymphotoxin (TNF-β), both of which are recognised by the same receptors. TNF receptors are of two types: 75 kDa (type 1) and 55 kDa (type 2).

IL-1 and TNF share many proinflammatory responses including, a) induction of fever, sleep & anorexia, b) mobilisation and activation of polymorphonuclear leukocytes, c) increase in adhesion molecule expression, d) activation of T-cells, B cells and NK cells, e) stimulation of production of other cytokines and f) tissue degeneration in chronic inflammation. Both TNF and IL-1 increase expression of many genes, via in part, by activation of nuclear factor-kappa B (NFκB) and activating protein-1 (AP-1). A naturally occurring IL-1 receptor antagonist (IL-1RA), a 17 kDa protein, competes with IL-1 for receptor binding, blocking IL-1 activity in vivo and in vitro and prevents death in animals administered with bacteria or LPS (Arend, 1993). Levels of IL-IRA enhance in patients with various infectious and inflammatory conditions. Thus, the balance between IL-1 and IL-IRA may contribute to the extent of an inflammatory response. Some other cytokines and growth factors also exert anti-inflammatory activity, which include, transforming growth factor β (TGF-β, which increases formation of extracellular matrix), IL-10 (has inhibitory effects on monocytes and macrophages, decreases cytokine and PG formation) and IFN-γ (has myelosuppressive activity and inhibits collagen synthesis and collagenase production by macrophages).

**Chronic inflammation**: Some microorganisms due to their cell wall components resist phagocytosis. Sometimes, macrophages are unable to engulf larger physical particles, which lead to persistent presence of an antigen. Such events lead to chronic inflammatory response, resulting in significant tissue damage. The continuous accumulation and activation of macrophages is the hallmark of chronic inflammation. Cytokines released by chronically active macrophages stimulate fibroblast proliferation and collagen production. Chronic inflammation often leads to granuloma, which is a tumour like mass, consisting of a central area of activated macrophages containing multinucleated giant cells formed by fusion of activated macrophages. TNF-α and IFN-γ play central role in the development of chronic inflammation. T_\text{H}-1 cells, NK cells and Tc cells
release IFN-γ, while activated macrophages release TNF-α. IFN-γ activates macrophages to exhibit increased expression of class II major histocompatibility complex (MHC) molecules, increased cytokine production and increased microbicidal activity. In Chronic inflammation, accumulation of activated macrophages is responsible for much of the tissue damage. These cells release various hydrolytic enzymes and ROS and RNI, which damage the surrounding tissue. IFN-γ also activates macrophages to produce more of TNF-α and also enhances TNF-α mRNA stability. Both cytokines work synergistically to initiate chronic inflammatory response. Chronic inflammation has been associated with a number of diseases in humans including rheumatoid arthritis, Crohn’s disease, ulcerative colitis, Graves disease, Hashimoto’s thyroiditis and diabetes mellitus (Girard & Springer, 1995), which are characterised by plump endothelium and the presence of mucin like cell adhesion molecules on its surface.

**Macrophages:**

Phagocytes (macrophages and neutrophils) make an important component of innate immunity as well as inflammatory response. The mononuclear phagocytic system consists of monocytes in blood and macrophages in tissues. The development of monocytes and macrophages takes place in bone marrow passing through the following steps:

Stem cell → committed stem cell → monoblast → promonocyte → monocyte (bone marrow) → monocyte (peripheral blood) → macrophages (tissues).

Monocyte differentiation in bone marrow proceeds rapidly, taking 1.5 to 3 days. Granules (azurophils and specific granules) are also formed in monocyte cytoplasm, just as in neutrophils. However in monocytes, they are fewer and smaller than their neutrophil counterparts, although their enzyme content is similar. Blood monocytes possess migratory, chemotactic, pinocytic and phagocytic properties and also possess the receptors for IgE-Fc domains & iC3B complement component. Monocytes undergo further differentiation (at least for a day) to become multifunctional tissue macrophages. However, monocytes represent the circulating macrophage population and are fully functional, able to change phenotype in response to factors encountered in specific tissue after...
Differentiation of a monocyte into a tissue macrophage involves a number of changes. These include:

a) the cell enlarges 5-10 folds.

b) intracellular organelles increase in number and complexity.

c) the cell acquires increased phagocytic ability.

d) the cell provides higher levels of hydrolytic enzymes.

e) the cell begins to secrete a variety of soluble factors.

Macrophages are dispersed throughout the body. Some take up residence in particular tissues, becoming ‘fixed’ macrophages, whereas others remain as ‘free’, motile and wandering macrophages. Resident macrophages are named according to their tissue location:

- Histiocytes - connective tissue
- Alveolar macrophages - lungs
- Kupffer cells - liver
- Mesangial cells - kidneys
- Microglial cells - brain
- Osteoclasts - bones
- Histiocytes, Langerhan's Cells - skin
- Free & Fixed macrophages - lymph nodes, spleen
- Fixed macrophages - bone marrow.

The macrophage population in a particular tissue may be maintained by three mechanisms:

a) influx of monocytes from circulation

b) local proliferation

c) biological turnover

Under normal steady state conditions, the renewal of tissue macrophages occurs via local proliferation, not by influx. Originally, it was thought that tissue macrophages are long living cells. Now, however it has been shown that depending on the type of tissue, their viability ranges between 6-16 days. Tissue macrophages are a population of ubiquitously distributed mononuclear
phagocytes responsible for homeostatic, immunological and inflammatory processes. These cells provide immediate defense against foreign elements prior to leukocyte immigration. Macrophages participate in both specific (via antigen presentation and IL-1 production) as well as non-specific (against bacterial, viral, fungal, neoplastic pathogens) immunity.

**Heterogeneity of Macrophages**

Macrophages isolated from different anatomical sites display a diversity of phenotypes and capabilities. It is suggested that macrophage heterogeneity arises from unique conditions within different specific tissue. For example, human breast milk macrophages express an antigen, not expressed on monocytes, alveolar macrophages and peritoneal macrophages (PMs). Macrophages from different tissues display heterogeneous functions, e.g. only a small number of PMs show 5'-nucleosidase activity whereas, immune elicitation of PMs, results in macrophage population, a high number of which exhibit 5'-nucleosidase activity, presumably by influx of monocytes. Production of macrophage lineage from bone narrow progenitors is normally controlled by M-CSF (macrophage-colony stimulating factor). In response to invasive stimuli and inflammation, monocyte number increases dramatically, so do the levels of M-CSF in serum. Levels of GM-CSF (granulocyte-macrophage CSF) also increase. Although, there appears to be an overlap of macrophage progenitors, which are able to respond to M-CSF & GM-CSF, but different structures and signal transduction mechanisms of the receptors for M-CSF & GM-CSF suggest that they initiate different differentiation pathways.

**Alveolar Macrophages**

AMs are the lung tissue resident macrophage (Hocking & Golde, 1979). AMs are known to differ from other tissue macrophages in a number of ways. These differences include the bioenergetics, expression of cell surface receptors, antigen presentation, bacterial killing and cytokine generation (Oren et al., 1963; Lipscomb et al., 1986; Nibbering et al., 1987; 1989; Rich et al., 1989; Nakamura et al., 1996). For example, human AMs express high levels of MHC-II where as opposite is found for PMs. AMs are the only macrophages residing in aerobic conditions. Due to the location, function and anatomy of lung tissue,
resident AMs are constantly exposed to infectious agents, allergens, and environmental pollutants and as a consequence they exist in a more activated state relative to resident peritoneal or any other tissue macrophages (Mbawuike & Herscowitz, 1988). AMs have a lobulated nucleus and a vacuolated cytoplasm containing numerous mitochondria and lysosomes (Pratt et al., 1971). The size of the cells varies between 12-40 μm. These cells are situated at the air tissue interface in alveoli and alveolar ducts. Several investigators have classified alveolar macrophages into different subpopulations (Zwilling et al., 1982; Holian et al., 1983; Chandler et al., 1986; Sandron et al., 1986a; 1986b) and the membrane receptor expression and cell functions such as phagocytosis and mediator release, have been shown to vary among these different subpopulations (Shellito & Kaltreider, 1984, Hance et al., 1985; Chandler & Fulmer 1987; Sibille et al. 1987). The distribution of AMs among the different subpopulations varies according to disease states. For example, in acute inflammation, an increased number of small monocyte like macrophages is observed, whereas chronic lung disorders are characterised by an increase in larger mature macrophages (Brannen & Chandler, 1988). In addition, each subpopulation can respond heterogeneously to different stimuli. Inspite of this heterogeneity, the main function is to contain an invader and prevent the damage to the surrounding delicate lung tissue.

Biological Functions of Macrophages

AMs perform vital functions like phagocytosis, antigen processing and presentation to T-cells, microbial killing by oxygen dependent and independent pathways by releasing free radicals, enzymes and clinical mediators like cytokines involved in the generation of immune response and inflammation.

The resident macrophages are immunologically quiescent, having low oxygen consumption, low levels of MHC-II expression and little or no cytokine production. However, they retain phagocytic, chemotactic and some proliferative capacity. The term ‘activated’ macrophages is reserved for the macrophages possessing specifically increased functional activity. In the first step in macrophage activation, there is enhanced MHC-II expression, antigen presentation, oxygen consumption and reduced proliferative capacity. The
agents that prime macrophages are IFN-γ (a product of stimulated T-helper cells), IFN-α, β, IL-3, GM-CSF, M-CSF & TNF-α. These primed macrophages respond to secondary stimuli to become fully activated. In this situation, cells are unable to proliferate, consume high oxygen, kill facultative parasites, lyse tumour cells, secrete maximum inflammatory mediators etc. These secondary signals are diverse and include LPS, heat killed G+ bacteria, yeast glucans, GM-CSF and phorbol esters (e.g. phorbol myristate acetate). The distinction between primed and activated macrophages is arbitrary depending on the stimulus used.

**Phagocytosis**

Macrophages are capable of ingesting and digesting exogenous antigens like whole microorganism, insoluble particles, and endogenous matter like injured and dead host cells, cellular debris, activated clotting factors. A number of anti-microbial cytotoxic substances are produced intracellularly by activated macrophages that can destroy phagocytosed material. The material within a membrane bound structure, phagosome, fuse with a lysosome to form phagolysosome. Lysosomes contain H₂O₂, oxygen free radicals, lysozyme, hydrolytic enzymes, which digest the ingested material. Phagocytosis can be through membrane receptors also. Macrophage membrane possesses receptors for certain antibodies and complement components, called opsonins. Any antigen coated with any antibody or complement component binds more readily to macrophage membrane through receptors and as a result phagocytosis is enhanced.

**Antigen Processing and Presentation**

Not whole of the antigen ingested by macrophages is degraded and eliminated by exocytosis. Phagocytosed antigen is degraded within the endocytote processing pathway into peptides, that associate with MHC-II molecules. These peptide-MHC-II complexes move towards the surface of macrophage membrane where they are presented to T-helper cells. This presentation of antigen to T-helper cells is the critical requirement for the activation of T-helper cell, a central event in the development of humoral and cell mediated immune responses.
**Antimicrobial and Cytotoxic Activities**

Macrophages are able to kill invading pathogen by oxygen dependent and oxygen independent pathways by releasing a number of antimicrobial and cytotoxic substances. More than one hundred of different substances central to the development of immune responses are secreted by activated macrophages. Some of these are shown in Table 2.2.

**Oxygen Dependent Killing Mechanisms**

Activated phagocytes produce a number of reactive oxygen and reactive nitrogen species that have potent anti-microbial activity. Activated phagocytes undergo a metabolic process 'respiratory burst' discovered by Baldridge & Gerard in 1933, which involves a burst of oxygen consumption (Iyer et al., 1961), that is caused by an “NADPH Oxidase” complex that assembles at the phagosomal membranes. Electrons are transferred from cytoplasmic NADPH to oxygen, generating superoxide anion.

**The Enzymes**

Phagocytes manufacture stupendous battery of reactive oxidants, used to kill invading microorganisms, by four enzymes – NADPH oxidase, superoxide dismutase (SOD), nitric oxide synthase (NOS) and myeloperoxidase (MPO) (Figure 2.1).

**NADPH oxidase** is a membrane bound enzyme that catalyses the production of superoxide anions ($O_2^-$) from $O_2$ and NADPH (Babior, 1999).

$$2O_2+NADPH \rightarrow 2O_2^-+NADP^++H^+$$

It is dormant in resting phagocyte but comes to life when cell is activated by any of the stimuli including bacteria and inflammatory polypeptides. Enzyme is composed of a number of subunits, which assemble on the membrane only on activation (Heyworth et al., 1991). The deficiency of any of the subunit renders the enzyme inactive and leads to chronic granulomatous disease (CGD).
Table 2.2: Secretory molecules of macrophages

<table>
<thead>
<tr>
<th>Category</th>
<th>Secretions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microbial and Cytotoxic</strong></td>
<td>Superoxide, hydrogen peroxide, hydroxyl radical, hypohalides, chloramines</td>
</tr>
<tr>
<td>Reactive oxygen intermediates</td>
<td></td>
</tr>
<tr>
<td>Reactive nitrogen intermediates</td>
<td>Nitric oxide, nitrates, nitrates</td>
</tr>
<tr>
<td><strong>Tumoricidal</strong></td>
<td>H₂O₂, NO, TNF-α, C3a, proteases, arginase, thymidine</td>
</tr>
<tr>
<td><strong>Tissue damaging</strong></td>
<td>H₂O₂, NO, TNF-α, neutral proteases</td>
</tr>
<tr>
<td><strong>Fever inducing</strong></td>
<td>IL-1, TNF-α, IL-6</td>
</tr>
<tr>
<td>Pyrogenic cytokines</td>
<td></td>
</tr>
<tr>
<td><strong>Inflammation Regulators</strong></td>
<td>Prostaglandins, prostacyclin, thromboxanes, leukotrienes</td>
</tr>
<tr>
<td>Bioactive lipids</td>
<td></td>
</tr>
<tr>
<td>Bioactive oligopeptides</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Complement components</td>
<td>C1-C5, factors B, D, P, I, H</td>
</tr>
<tr>
<td>Cytokines</td>
<td>IL-1, IL-6, IL-8, TNF-α, IFN-γ, MIP-1, 2, 3, M-CSF, G-CSF, GM-CSF, PDGF</td>
</tr>
<tr>
<td>Neutral proteinases</td>
<td>Elastase, collagenase, angiotensin convertase</td>
</tr>
<tr>
<td>Acid hydrolases</td>
<td>Acid proteases, peptidases, lipases, lysozyme, glycosidases, ribonucleases, phosphatases, sulphatases</td>
</tr>
<tr>
<td>Stress proteins</td>
<td>Heat shock proteins</td>
</tr>
<tr>
<td><strong>Tissue reorganisation</strong></td>
<td>Elastase, collagenase, hyaluronidase, regulatory growth factors, fibroblast GF, TGF-α and TGF-β, angiogenesis factors</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td>Apolipoprotein E, IL-1 inhibitors, thymidine, uracil, neopterin</td>
</tr>
</tbody>
</table>
The patients with CGD are highly susceptible to bacterial and fungal infections (Forrest et al., 1988) as they are unable to manufacture any microbicidal oxidants. **Superoxide dismutase** catalyses the conversion of $O_2^-$ to $H_2O_2$ and $O_2$ by dismutation reaction.

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

$H_2O_2$ provided by SOD becomes a substrate for **MPO** as MPO catalyses the oxidation of halide ions to hypohalous acids at the expense of $H_2O_2$ (Hampton et al., 1998).

$$H_2O_2 + Cl^- \rightarrow OCl^- + H_2O$$
The enzyme can oxidise any of the halide ions (Cl\(^-\), Br\(^-\), I\(^-\)) and in addition of SCN\(^-\), a pseudohalide that is abundant in saliva (Furtmullar \textit{et al}., 1998). OCI\(^-\) is the principal product, because of high concentrations of Cl\(^-\) in body fluids.

**NOS** catalyses the production of NO\(^-\) from arginine, oxygen and NADPH (Marletta, 1994).

Arginine + oxygen + NADPH $\rightarrow$ NO + citrulline + NADP\(^+\)

This enzymatic oxidation of amino acid, L-arginine, is catalysed by 3-different isoforms of NOS (Moncada & Higgs, 1993; Gaston \textit{et al}., 1994): the neuronal isoform nNOS, the endothelial isoform eNOS and a mitogen inducible isoform iNOS, which is especially found in macrophages. In contrast to iNOS, nNOS and eNOS are constitutively expressed in certain characteristic cell types (neuronal and endothelial). However, it is now known that nNOS and eNOS can also be induced under certain physiological conditions and iNOS may function as "constitutive" enzyme in some cells (Gho \textit{et al}., 1995). Thus, the nomenclature of NOS as constitutive or inducible is a bit misleading. As denoted by its prefix, nNOS was originally purified and cloned from neuronal tissues. However, nNOS is now known to be much more widely distributed, with an important level of expression in skeletal muscle. iNOS, originally purified and cloned from an immonoactivated cell line, has since been identified in myriad mammalian tissues. iNOS expression has been studied in cells as diverse as cardiac myocytes, glial cells, vascular smooth muscle cells. The human genes for NOS isoforms are officially categorised in the order of their isolation and characterisation. The human genes encoding nNOS, iNOS and eNOS are termed as NOS-1, NOS-2 and NOS-3 respectively (Michel & Feron, 1997). Recently, a mitochondrial NOS (mtNOS) has been reported to be present in the inner mitochondrial membrane (Ghafourifar & Richter, 1997; Tatoyan & Giulivi, 1998). The same NOS isoform may play entirely distinct biological roles when expressed in different tissues. The pathways outlined in one tissue may not be pertained when the same isoform is expressed in a different cell.

There are important general biochemical features shared in common by the different NOS isoforms (Marletta, 1994; Nathan & Xie, 1994). The overall amino acid sequence identity for the three human NOS isoforms is 55%, with
particularly strong sequence conservation in the protein regions importantly involved in catalysis (Michel et al., 1995). The three isoforms share a similar catalytic scheme, involving the five-electron oxidation of the terminal guanido nitrogen of L-arginine to form NO\textsuperscript{-} and citrulline. This complex reaction involves molecular oxygen and NADPH as a cosubstrate with numerous other redox cofactors including enzyme bound heme, reduced thiols, FAD, FMN and tetrahydrobiopterin (Michel & Feron, 1997). For all three isoforms, NO synthesis depends on the enzyme’s binding to ubiquitous calcium regulatory protein calmodulin. For full activity of nNOS and eNOS, and their binding to calmodulin, increases in intracellular calcium concentrations [Ca\textsuperscript{2+}] are required. In contrast, iNOS appears to bind with calmodulin with extremely high affinity even at low [Ca\textsuperscript{2+}], which is a characteristic of resting cells. Thus, the intracellular activity of eNOS and nNOS may be closely modulated by transient changes in Ca\textsuperscript{2+} concentrations, whereas activity of iNOS in immunoactivated cells is no longer temporally regulated by intracellular calcium transients (Nathan & Xie, 1994). Inflammatory macrophages possess cytotoxic and antimicrobial effector functions based on their ability to produce nitric oxide (Jorens et al., 1995; MacMicking et al., 1997) by iNOS. The fundamental importance of this metabolic pathway in murine macrophages, as a key defense element in various infections as well as its role in diverse settings of immunopathology, is firmly established (Kroncke et al., 1995; Adler et al., 1997). The stimuli which induce iNOS include inflammatory cytokines (IL-1, TNF-\alpha), LPS, phorbol esters etc. (Nakayama et al., 1992; Robbins et al., 1994; Kristof et al., 1998). Once expressed, the inducible enzyme generates significantly larger and sustained amounts of NO than does the constitutive form (Clancy & Abramson, 1995), which via inappropriate vasodilation, causes septic organ failure (Stewart et al., 1995; Ullrich et al., 1999).

**The Oxidants**

The oxidants produced by activated macrophages include superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide, singlet oxygen, peroxynitrite etc. Table 2.3 summarises some of their characteristics.
Table 2.3: Some characteristics of reactive oxygen & nitrogen derivatives

<table>
<thead>
<tr>
<th>Species</th>
<th>Symbol</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide radical</td>
<td>O₂⁻</td>
<td>Good reductant, poor oxidant</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>OH</td>
<td>Extremely reactive (addition, abstraction and electron transfer reactions), very low diffusion distance</td>
</tr>
<tr>
<td>Perhydroxyl radical</td>
<td>HO₂⁻</td>
<td>Stronger oxidant and more lipid soluble than superoxide, may initiate lipid peroxidation</td>
</tr>
<tr>
<td>Peroxyl radical</td>
<td>ROO</td>
<td>Low oxidising ability relative to OH but greater diffusibility</td>
</tr>
<tr>
<td>Alkoxy radical</td>
<td>RO</td>
<td>Intermediate in their reactivity with lipid between ROO &amp; OH</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>H₂O₂</td>
<td>Oxidant but reactions with organic substrates are sluggish, high diffusion capability</td>
</tr>
<tr>
<td>Singlet oxygen</td>
<td>O₂</td>
<td>Powerful oxidising agent</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>NO</td>
<td>Freely diffusible, having physiological &amp; pathophysiological roles</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>ONOO⁻</td>
<td>Powerful cytotoxic and antimicrobial agent</td>
</tr>
</tbody>
</table>

Superoxide and Hydrogen Peroxide

A variety of soluble and particulate stimuli induce extracellular superoxide production (Klebanoff, 1992; Thomas et al., 1992; Segal & Abo, 1993; Robinson & Badwey, 1995). Most of the superoxide formed is dismutated to H₂O₂ by superoxide dismutase. Exogenously generated superoxide does not kill bacteria directly (Klebanoff, 1974; Babior et al., 1975; Rosen & Klebanoff, 1979). Hydrogen peroxide is bactericidal only at high concentrations (Imlay & Linn, 1986; Hyslop et al., 1995). A variety of secondary oxidants have been proposed to account for the destructive capacity of phagocytes.
Hydroxyl Radical and its Secondary Products

One of the most reactive free radical oxidants is hydroxyl radical (OH), with extremely short half life (Table 2.4). In biological systems, OH is generated by the reaction between H2O2 and iron/copper in low valence state (Halliwell & Gutteridge, 1986).

Table 2.4: Life times of reactive oxygen species

<table>
<thead>
<tr>
<th>Species</th>
<th>Symbol</th>
<th>Half life at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide radical</td>
<td>O2⁺</td>
<td>1 micro sec</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>OH⁺</td>
<td>1 nano sec</td>
</tr>
<tr>
<td>Singlet oxygen</td>
<td>¹O₂</td>
<td>1 μ sec</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>NO⁻</td>
<td>0.1 to 5.6 sec</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>ONOO⁻</td>
<td>1.6 sec</td>
</tr>
<tr>
<td>Alkoxyl radical</td>
<td>RO⁻</td>
<td>1 μ sec</td>
</tr>
<tr>
<td>Peroxy radical</td>
<td>ROO⁻</td>
<td>0.01 to 7 sec</td>
</tr>
<tr>
<td>Molecular oxygen</td>
<td>O₂</td>
<td>&gt;10² sec.</td>
</tr>
</tbody>
</table>

Adapted from Pryor, 1984; Florence, 1990; Kelm & Schrader, 1990; Rubbo et al., 1996.

H₂O₂+Fe²⁺→OH⁺+OH⁻+Fe³⁺

Hydroxyl radical further reacts with molecules that contain no unpaired electrons, giving another free radical product, e.g.

=C–H + OH → =C–H₂O

This type of reaction gives rise to a very large variety of free radical species, with the unpaired electron resting on carbon, oxygen, nitrogen or sulphur. Many of these secondary radicals further react with other compounds to produce yet other free radicals. Hence, a free radical chain starts. The chain terminates only when a free radical reacts with another molecule with unpaired electron – a free radical or a transition metal, for example

=C + C = → =C–C=

There are two potential mechanisms for hydroxyl radical production by phagocytes: the superoxide driven Fenton reaction between H₂O₂ and metal catalyst and by the reaction of HOCl with superoxide (Candeias et al., 1993).
Myeloperoxidase limits the reaction further, even if iron is available, by consuming H$_2$O$_2$ (Winterbourne, 1986). Hydroxyl radicals kill bacteria (Wolcott et al., 1994), but they have limited radius of action. Even in the confined space of phagosome, most hydroxyl radicals are likely to react with other targets before reaching the bacterium. Czapski et al., 1992, have shown that hydroxyl radicals generating systems are more toxic to bacteria in the presence of chloride, attributing the toxic effects to HOCl. HOCl is the most bactericidal oxidant known to be produced by phagocytes (Klebanoff, 1968). Many species of bacteria are readily killed by myeloperoxidase/H$_2$O$_2$/chloride system (Albrich & Hurst, 1982). Bacterial targets for HOCl are Fe/S proteins, membrane transport proteins, ATP generating systems, origin of replication (OriC) for DNA synthesis (Albrich et al., 1986; McKenna & Davies, 1988; Barrette et al., 1989; Rosen et al., 1990; Rosen & Michel, 1998). HOCl reacts with amines to generate chloramines (Thomas & Learn, 1991; Carr & Winterbourne, 1997), which are also bactericidal (Grisham et al., 1984; Belike et al., 1989). Chloramines are oxidising species. Their reactivity varies with their lipid solubility. Chloramine (NH$_2$Cl) formed by reaction of HOCl with ammonia is much lipid soluble and more toxic than HOCl itself. Similarly, putrescine chloramine (H$_2$N(CH$_2$)$_4$ NHCl) is even more lipid soluble and even more toxic than chloramines (Grisham et al., 1984). Conversely, taurine chloramine (SO$_3$$^-$ (CH$_2$)$_2$ – NHCl) is soluble in water and shows little toxicity (Wright et al., 1985). Infact, taurine appears to be used by neutrophils as a protective antioxidant, slowing the rate at which neutrophils are killed by their own HOCl (Green et al., 1991). The reaction of HOCl with amino acids leads to aldehydes through chloramines.

$$R-CHNH$_2$COOH+HOCl\rightarrow R-CHNHCl-CO0H\rightarrow R-CHO+CO$_2$+NH$_4$+Cl'$$

Aldehydes are another class of very reactive molecules. Furthermore O$_2$$^\cdot$ reacts with HOCl, showing intersection of free radicals and oxidised halogens (Kettle & Winterbourne, 1994). Altogether, these species form a battery of lethal oxidising agents; oxidising halogens being probably the most important microbicidal oxidants.
**Singlet Oxygen**

The much more reactive form of oxygen is singlet oxygen ($^{1}O_2$), in which two electrons are paired, produced by the reaction (Kanofsky *et al.*, 1984).

$$H_2O_2+OCl^{-} \rightarrow ^{1}O_2+H_2O+Cl^{-}$$

$^{1}O_2$ is likely to be responsible for some of the damage inflicted by phagocytes on their targets.

**Nitric Oxide**

NO, a freely diffusible intercellular messenger (Table 2.3), with a half life of 0.1-5.6 sec (Table 2.4), is a pleiotropic molecule that has been implicated in a variety of biological phenomena such as vasodilation, platelet aggregation, synaptic transmission, macrophage cytotoxicity and cell death (Moncada *et al.*, 1991; Gaston *et al.*, 1994). A variety of mammalian cells are known to produce NO which includes vascular endothelium, smooth muscle cells, macrophages, neutrophils, platelets and pulmonary epithelium (Moncada & Higgs, 1991; Rubbo *et al.*, 1996; Mayer & Hemmens, 1997). NO is a labile molecule, which may carry out important biological roles, both within the cells in which it is synthesised as well as in interactions with nearby cells and molecules (Ignarro, 1990; Stamler, 1994). The NO formed diffuses to nearby smooth muscle cells, in which it reacts with the ferrous ion in the heme group of enzyme guanylate cyclase, resulting in its activation and enhanced production of cGMP from GTP (Moncada & Higgs, 1993). Cyclic GMP then interacts with GMP dependent kinases, cGMP regulated cyclic nucleotide phosphodiesterases and cGMP regulated ion channels. Cyclic GMP dependent kinases stimulate Ca$^{2+}$-ATPase and eventually lower intracellular calcium levels, thus mediating smooth muscle cell relaxation (Cornwell & Lincoln, 1993). Nitric oxide can also bind to non-heme iron (Reif & Simmons, 1990). Such interactions have been implicated in the control of translation of some polypeptides (Weiss *et al.*, 1993). NO has also been reported to help regulate neurotransmitter release in neuronal tissues (Dawson & Snyder, 1994). Nitric oxide itself acts as a neurotransmitter (Bult *et al.*, 1990) and controls the smooth muscle relaxation (Snyder & Bredt, 1991). Nitric oxide also inhibits platelet aggregation by a cGMP dependent mechanism, and synergises with prostacyclins, which inhibit platelet aggregation by
increasing the concentration of cAMP (Radomski & Monocada, 1991). Thus, platelet aggregation \textit{in vivo}, may be regulated by platelet derived NO as well as NO and prostacyclins released from vascular endothelium. Nitric oxide may also be involved in the interaction of leukocytes with vessel walls, since it inhibits leukocyte activation (Kubes \textit{et al.}, 1991). It also inhibits the proliferation of smooth muscle cells, hence participates in general homeostatic control of the vasculature (Garg & Hassid, 1989). Nitric oxide produced by endothelium is involved in the physiological regulation of vascular tone in different organs (Kirkeboen \textit{et al.}, 1992,1994; Naess \textit{et al.}, 1992) and thereby plays important role in regulation of blood pressure and distribution of blood flow (Calver \textit{et al.}, 1993).

\textbf{Role of NO in host defense}

Macrophages, monocytes, neutrophils, mast cells, all synthesise NO by iNOS during stimulation with different agents (Salvemini \textit{et al.}, 1989; Davies \textit{et al.}, 1995). The immunological induction of NO synthesis in macrophages is an important part of host defense (Stuehr \textit{et al.}, 1989). NO synthesis by activated macrophages leads to non-specific cytotoxicity against bacteria, protozoa and tumour cells (Adams \textit{et al.}, 1990; Liew \textit{et al.}, 1990; Jagannath \textit{et al.}, 1998). This cytotoxicity has been attributed to the cytotoxic NO derivatives like peroxynitrite (Mulligan \textit{et al.}, 1992). NO inhibits cell replication by inactivation of enzymes such as ribonucleotide reductase (Hibbs \textit{et al.}, 1988; Nathan, 1992). Mitochondrial electron transport chain is impaired by NO by inhibiting cytochrome oxidase activity by competing with oxygen; by inhibiting electron transfer between cytochrome b and c and increasing the mitochondrial production of $O_2^\cdot$ and by inhibiting electron transfer and NADH-dehydrogenase function in complex I (Hibbs \textit{et al.}, 1988; Nathan, 1992; Boveris \textit{et al.}, 2000). It has been postulated that NO binds to the iron-sulphhydryl groups of the mitochondrial respiratory chain components, resulting in cell death. Although, desirable in terms of inhibition in bacterial and tumour growth, NO and its derivatives may cause damage to normal tissue when produced in excess.

Nitric oxide modulates inflammation and oxidative stress by reacting with metalloproteins, protein sulphydryls and oxygen derived free radicals (Rubbo \textit{et
The reactivity of NO with ROS leads to a diversity of both toxic and cytoprotective effects. Nitric oxide is more likely to act as prooxidant when concentration of superoxide is higher than or equal to NO via the reaction of these species to form a potent oxidant, peroxynitrite (Huie & Padjama, 1993). Superoxide actually accelerates the 'destruction' of NO and thus inhibits NO mediated vascular relaxation. This inactivation of NO by O_2^- was prevented by SOD (Gryglewski et al., 1986). NO reacts with O_2^- to give peroxynitrite and its conjugate acid peroxynitrous acid (HNOOH). The rate constant for this reaction is 2x10^{10} \text{M}^{-1}\text{Sec}^{-1}, faster than SOD catalysed dismutation of O_2^- (Gutierrez et al., 1996). Peroxynitrite is a potent oxidant with a half life of 1.6 sec at neutral pH (Rubbo et al., 1996) (Table 2.4). Peroxynitrous acid reacts by two pathways, by yielding nitrate (NO_3^-) without forming strong oxidant intermediates or by forming hydroxyl radical and nitrogen dioxide (NO_2), a potent oxidant (Beckman et al., 1990). Peroxynitrite reactivity is also influenced by CO_2, with the formation of nitrosoperoxocarbonate (ONOOCO_2^-) intermediate. Consequently, CO_2 stimulates peroxynitrite decay and enhances peroxynitrite-mediated nitration of molecules by 2 fold (Radi et al., 1999).

**Oxygen Independent Killing Mechanisms**

Activated macrophages synthesise lysozyme and other various hydrolytic enzymes whose degradative activities do not require oxygen. Activated macrophages also produce a group of antimicrobial and cytotoxic peptides, commonly known as defensins (Table 2.5). These are cysteine rich cationic peptides, each 29-35 amino acid long, which forms a circular molecule that is stabilised by intramolecular disulphide bonds. These circularised defensive peptides have been shown to form ion permeable channels in bacterial cell membranes. Defensins can kill a variety of bacteria. TNF-\(\alpha\) produced by activated macrophages has a variety of effects and is cytotoxic. IL-1 activates lymphocytes, IL-6 and TNF-\(\alpha\) promote fever by affecting thermoregulatory centre in hypothalamus. Complement proteins secreted by macrophages, assist in eliminating foreign pathogens. Hydrolytic enzymes contained in lysosomes, contribute to inflammatory response and sometimes, extensive tissue damage.
Table 2.5: Factors responsible for oxygen independent killing mechanisms of macrophages

<table>
<thead>
<tr>
<th>Factor</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defensins</td>
<td>Antimicrobial, cytotoxic peptides</td>
</tr>
<tr>
<td>IL-1</td>
<td>Promotes inflammatory responses and fever</td>
</tr>
<tr>
<td>IL-6, TNF-(\alpha)</td>
<td>Promote innate immunity and elimination of pathogens</td>
</tr>
<tr>
<td>Complement Proteins</td>
<td>Promote inflammatory response and elimination of pathogens.</td>
</tr>
<tr>
<td>Hydrolytic Enzymes</td>
<td>Promote inflammatory response</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>Activates cellular genes, resulting in production of antiviral proteins</td>
</tr>
<tr>
<td>TNF-(\alpha), GM-CSF</td>
<td>Kill tumour cells</td>
</tr>
<tr>
<td>G-CSF, M-CSF</td>
<td>Promote inducible hematopoiesis</td>
</tr>
</tbody>
</table>

The inflammatory secretions as well as other cytokines such as IL-2, IL-6, and IL-8 all are under the control of a common genetic transcription factor known as NF\(\kappa\)B (Blackwell & Christman, 1997). NF\(\kappa\)B is heterodimeric protein complex containing two members of rel family of proteins (Baldwin, 1996). In the cytoplasm of most cells, the heterodimeric NF\(\kappa\)B complex is bound to an inhibitory factor and \(\kappa\)B (Blackwell & Christman, 1997; Whiteside et al., 1997). Upon stimulation or challenge with foreign substance (Zhu et al., 1996; Baeuerle & Baichwal, 1997; Barnes & Karin, 1997; Blackwell and Christman, 1997), \(\kappa\)B is separated and undergoes proteolytic degradation. Free NF\(\kappa\)B heterodimer then translocates into the nucleolus where it binds to the kappa B promoter regions of selected inflammatory genes and upregulates or inhibits the transcription of these genes (Siebenlist et al., 1994; May & Ghosh, 1998; Sha, 1998).

**Role of Macrophages in Inflammatory Diseases**

Inordinate and aberrant generation of ROS is widely incriminated in the pathogenesis of tissue injury (Halliwell & Gutteridge, 1999a). Oxidative stress is now thought to make significant contribution to all inflammatory diseases including arthritis (McCord, 1974; Vaille et al., 1990), lupus erythematosus
(Mohan & Das, 1997), Ischemic injuries to heart, brain and intestine (Parks et al., 1982; Omar & McCord, 1991; Baker et al., 1998), hemochromatosis (Houglum et al., 1997), AIDS (Flores et al., 1993), emphysema (Wallaert et al., 1993), gastric ulcers (Davies et al., 1992), hypertension (Kerr et al., 1999), neurological diseases (multiple sclerosis, Parkinson's disease, Alzheimer's disease, muscular dystrophy, amyotrophic lateral sclerosis) (Cohen, 1984; Toshniwal & Zarling, 1992; Lyras et al., 1997; Ragusa et al., 1997; Aguirre et al., 1999) and alcohol or smoking related diseases (Dianzani, 1985; Asami et al., 1997).

ROS commonly incriminated in the pathogenesis of tissue injury include $O_2^-$, OH and $H_2O_2$. Recently, NO & ONOO$^-$ are also recognized for their capacity to induce oxidant injury (Halliwell & Gutteridge, 1999b). It has been suggested that reactive oxygen and nitrogen species produced by activated phagocytes are responsible for the genetic lesions (mutations and sister chromatid exchanges) caused in bacteria by human phagocytes (Weitzman & Stossel, 1981; Weitzman et al., 1985; Liu & Hotchkiss, 1995; Wiseman & Halliwell, 1996). Such genetic lesions caused by inflammatory phagocytes may play role in carcinogenesis (Wiseman & Halliwell, 1996). With the continuous exposure to environmental agents like pollutants and pathogens, alveolar macrophages remain in activated state. Macrophage mediators, that can influence airway tone and inflammation, include histamine releasing factor (Schulman et al., 1985), eicosanoids and prostaglandins (Ranklin et al., 1984; Murray et al., 1986), platelet activating factor (Salem et al., 1990), hydrolytic enzymes (Tonnell et al., 1983) and ROS & RNI (Wallaert et al., 1990; Gaston et al., 1994). ARDS is an inflammatory condition caused by leakage of fluids into alveoli that results from damage to endothelium of pulmonary capillaries. ARDS may be caused by inhalation of toxic gases, which directly damage the endothelium. There is a lot of evidence suggesting that some of the damage to endothelium in pulmonary capillaries is the result of oxidants released by activated phagocytes (Chaquot et al., 1998). Evidence about the role of phagocyte derived oxidants in ARDS comes from some of the studies including endotoxic shock models (Gonzalez et al., 1995; Bloomfield et al., 1997), which are characterised by sequestration of neutrophils in lungs (Wieland et al., 1986)
and such effects were diminished by antioxidants, SOD and catalase (Till et al., 1982). Role of NO, in ARDS, in the absence of oxidants (in gp91<sub>PHOX</sub> knockout mice) has been demonstrated (Kubo et al., 1996). Several studies have shown that activated phagocytes (neutrophils and macrophages) are able to accomplish the oxidation of low density lipoprotein (LDL) (Abdalla et al., 1994; Katsura et al., 1994), which is important in the development of atherosclerotic lesion. Neutrophils inflict damage to reperfused tissue by free radical production (Hansel, 1995; Ambrosi & Tritto, 1999). Activated neutrophils can induce mutations in DNA of cells exposed to them, causing strand breaks and all four base modifications (Shacter et al., 1988; Jackson et al., 1996). Exposure to neutrophil-oxidants, led to the formation of transformants, which when injected into mice led to benign and malignant tumours (Tamatani et al., 1999). Another route by which neutrophils can promote carcinogenesis is via oxidations of procarcinogens. For example, oxidation of benzopyrene 7,8, dihydrodiol and 2-amino-flourene by activated neutrophils led to the production of DNA alkylating agents (Trush et al., 1985; Shen et al., 1990). Presence of activated macrophages, neutrophils, carbonylated and nitrated proteins in synovial fluid of inflamed joints led to the conclusion that oxidants production by activated phagocytes contribute to arthritis (Schmitz et al., 1987; Chapman et al., 1989; Kaur & Halliwell, 1994). Enhanced production of O<sub>2</sub>· by macrophages present in rheumatoid arthritis patients, indicates that ROS produced by phagocytes are important mediators in the pathogenesis of rheumatoid arthritis (Biemond et al., 1986; Dulray et al., 1988; Dewar & Harth, 1994). Role of oxidative stress has been demonstrated in acute renal failure and Alzheimer’s disease (Nath & Norby, 2000; Pratico & Delanty, 2000), but there are conflicting reports about whether the free radical injury is the cause or consequence of neurodegeneration in Alzheimer’s disease. This ubiquitous involvement of ROS in disease processes reflects the far ranging pathobiologic effects of ROS on cellular viability, growth and proliferation, tissue repair and regeneration, inflammatory and immune responses (Dalton et al., 1999; Allen & Tresini, 2000).
Biological Damages Caused by Oxidative Stress

Consistent exposure of organisms to one or more ROS generating systems leads to the damage to lipids, proteins, carbohydrates and nucleic acids (Halliwell & Gutteridge, 1999b).

Lipid Peroxidation

Lipid peroxidation appears to be a highly significant consequence of oxidative stress. Studies beginning in 1950s, provided good evidence that several halogenated hydrocarbons exert some or all of their toxic effects by stimulating lipid peroxidation in vivo. Both isolated polyunsaturated fatty acids (PUFAs) and those incorporated into lipids are readily attacked by free radicals, becoming oxidised into lipid peroxides. The greater the number of double bonds in a fatty acid (FA) side chain, the easier is the removal of hydrogen atom. Removal of hydrogen leaves behind an unpaired electron on carbon atom, resulting in carbon centred lipid radical, which can have several fates; the most likely one in aerobic cells is to undergo molecular rearrangement followed by reaction with O₂ to give peroxyl radical. Peroxyl radicals either react with each other, if they happen to meet, or they attack membrane proteins. They are also capable of abstracting hydrogen atoms from adjacent fatty acid side chains, so propagating the chain reaction of lipid peroxidation. Hence a single initiating event can result in the conversion of hundreds of FA side chains into lipid hydroperoxides. Length of the propagation time depends on many factors; a) lipid/protein ratio (the chance of a lipid radical reacting with a membrane protein will increase with an increase in protein content), b) the fatty acid composition, c) the oxygen concentration and d) the presence of chain breaking antioxidants (A-H) within the membrane, which interrupts the chain by providing an easily donatable hydrogen to lipid peroxy radical. The resulting antioxidant-derived radical (A) a) might react with another LO₂ molecule, b) may disappear harmlessly (by dimerisation to A₂), c) could be converted back to A-H by reaction with another molecule.

$$A{-}H + LO_2 \rightarrow LO_2H + A$$

The most important, but by no means the only, chain breaking antioxidant in human lipids is α-tocopherol (Burton & Ingold, 1989). Evidence exists that the
α-tocopherol radical can be converted back to tocopherol by reduction with ascorbic acid at the surface of biological membranes (Burton et al., 1990). The occurrence of lipid peroxidation in biological membranes causes impairment of membrane functioning, changes in fluidity, inactivation of membrane bound receptors and enzymes and increased non-specific permeability to ions such as Ca\textsuperscript{2+} (Slater, 1984; Jain, 1985; Comporti, 1987; Burton & Ingold, 1989; Steinberg et al., 1989). For example, deformation of red blood cells after exposure to peroxides causes them to become leaky to K\textsuperscript{+} ions (Sugihara et al., 1991).

Highly reactive OH\textsuperscript{-} can attack all biological molecules including membrane lipids. Attack by OH\textsuperscript{-} can initiate lipid peroxidation. Several transition metal salts react with H\textsubscript{2}O\textsubscript{2} to form OH but most attention to the OH generation in vivo has been paid to iron (Minotti & Aust, 1987; Halliwell & Gutteridge, 1990).

Initiation of lipid peroxidation in vivo may involve another potential pathway i.e. MPO. MPO is a membrane protein present in neutrophils, monocytes and certain populations of tissue macrophages (Savenkova et al., 1994; Schmitt et al., 1999; Podrez et al., 1999). Leukocyte activation in whole plasma has long been appreciated as a physiological mechanism for promoting peroxidation of endogenous plasma lipids (Frie et al., 1988). On phagocyte activation in peripheral tissues and fluids, MPO is secreted into the extracellular milieu and into the phagolysosome, where it uses H\textsubscript{2}O\textsubscript{2} generated during respiratory burst and halide ions (most likely Cl\textsuperscript{-}) and hypochlorous acid are produced (Harrison & Schultz, 1976). In addition to halides, pseudohalide thiocyanate (SCN\textsuperscript{-}), various organic and inorganic components found in plasma also serve as naturally occurring substrates for MPO (Van Dalen et al., 1997), generating reactive oxidants and diffusible radical species (Kettle & Winterbourn, 1997; Podrez et al., 2000). Although lipid peroxidation and lipid derived signalling molecule formation are believed to be critical in atherosclerosis and other inflammatory disorders, yet the pathways responsible for these processes in vivo are not fully established. Zhang et al., 2002, have shown that MPO is a major enzymatic catalyst for promoting lipid peroxidation by activated phagocytes in plasma.
Lipid peroxidation acts as a major source of cytotoxic products such as aldehydes produced from the decomposition of lipid hydroperoxides (Esterbauer et al., 1990). These aldehydes are capable of forming protein crosslinks that can cause damage to membrane proteins, inactivation of membrane receptors and enzymes (Dean et al., 1986). Products of lipid peroxidation react with critical biomolecules that may have a key role in the development of certain pathological states (Ames et al., 1982; Basu & Marnett, 1984; Berliner & Heinecke, 1996; Chisolm et al., 1999; Podrez et al., 2000). For example, several aldehydes including malondialdehyde (MDA) react with nucleic acids, thus probably contributing to mutagenesis and carcinogenesis (Basu et al., 1981; Siu & Draper, 1982; Basu & Marnett, 1983).

Oxidation of Proteins and Carbohydrates

Some of the common ROS generating systems known to modify proteins are shown in Figure 2.2.

These include a number of environmental factors such as irradiation, pollutants in the atmosphere (Ozone, \( \text{N}_2\text{O}_5 \), \( \text{NO}_2 \), cigarette smoke) and simple by products.
of normal metabolic processes such as autooxidation of reduced forms of electron carriers (NADPH, reduced flavins, cytochrome $P_{450}$), inflammatory reactions, NO, oxidase catalysed reactions, lipid peroxidation and metal catalysed reactions. In contrast to repair of oxidative damage to nucleic acids by efficient repair mechanisms, damage repair to proteins is limited to the reduction of oxidised derivatives of the S-containing amino acid residues (Stadtman & Levine, 2000). Repair of other kinds of protein oxidation has not been demonstrated. Instead, damaged proteins are subjected to degradation by various endogenous proteases including cathepsin C, calpain, trypsin and especially 20S proteasome (Rivett, 1986; Grune et al., 1996). The activity of these proteases is further controlled by diverse regulatory factors including concentration of enzyme substrate, ubiquitination, and various inhibitors. Presence of a battery of enzymatic and non-enzymatic antioxidants also protects the oxidative damage to proteins. Hence extent of protein oxidation is a complex function of prooxidant/antioxidant activities and accumulation of oxidised forms of proteins depends on concentrations/activities of the proteases that degrade the oxidised proteins. Virtually all kinds of amino acid residues of proteins are potential targets for oxidation by OH and Fe(II) (Huggins et al., 1993; Neuzil et al., 1993). At physiological conditions (low concentrations of Cu and Fe ions and $H_2O_2$), protein damage is limited to the modification of amino acid residues at metal binding sites on the proteins. So under normal physiological conditions, oxidation of proteins is a site specific process by which binding of Fe(II) and Cu(I) to metal binding sites is followed by reaction with peroxides to generate reactive species ($OH$, $RO$, perferryl radicals), which will react preferentially with amino acid residues at metal binding site (Chevion, 1988; Stadtman, 1990). ROS mediated oxidation to proteins leads to the conversion of amino acids to their different derivatives (Table 2.6). Of particular importance is the formation of carbonyl derivatives. Carbonyl groups formed may further react with $\alpha$-amino group of lysine residues on the same or other protein to form intra or inter molecular protein crosslinks. Some of such derivatives are not only resistant to proteolytic degradation by proteasome but may also inhibit the ability of the proteasome to degrade other oxidised forms of the proteins (Friguet et al.,
1994a, b). This may further contribute to accumulation of oxidised forms of proteins during aging and age related diseases. Elevated levels of oxidised proteins have been reported in a number of age related diseases. An increase in the carbonyl content of proteins is associated with Alzheimer’s disease (Hensley et al., 1995; Smith et al., 1998), Parkinson’s disease (Alam et al., 1997), diabetes (Uchida et al., 1998), rheumatoid arthritis (Chapman et al., 1989), muscular dystrophy (Murphy & Kehrer, 1989), cataractogenesis (Garland et al., 1988), induction of renal tumours (Uchida et al., 1995), bronchopulmonary dysplasia (Gladstone & Levine, 1994), chronic ethanol ingestion (Vendemiale et al., 1998), acute CCl₄ toxicity (Comporti, 1998), amyotrophic lateral sclerosis (Bowling et al., 1993) and progerias (Oliver et al., 1987). In some of the diseases, more than one kind of protein oxidation has been demonstrated.

### Table 2.6: Derivatives of amino acids produced as a result of oxidation

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>2-oxohistidine</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Kynurenine or N-formyl kynurenine</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Dihydroxy derivatives</td>
</tr>
<tr>
<td>Methionine</td>
<td>Methionine sulfoxide or methionine sulphone derivatives</td>
</tr>
<tr>
<td>Leucine/Valine</td>
<td>Leucine/valine hydroxy derivatives</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Disulphide derivatives</td>
</tr>
<tr>
<td>Lysine/Arginine/Proline</td>
<td>Carbonyl derivatives</td>
</tr>
</tbody>
</table>

Derived from Garrison, 1987; Amici et al., 1989; Brodie & Reed, 1990; Uchida et al., 1990; Uchida & KawaKishi, 1993; Pryor & Uppu, 1993; Huggins et al., 1993; Dean et al., 1993; Heinecke et al., 1993; Kikugawa et al., 1994; Vogt, 1995; Berlett et al., 1996.

### Oxidative DNA Damage

DNA damage by ROS is an important intermediate in the pathogenesis of human diseases such as cancer and aging (Ames, 1987; Guyton & Kensler, 1993). ROS induced DNA damage products are both mutagenic and cytotoxic (Wallace, 1994). Several studies have characterised ROS mediated DNA
damage, which includes single and double strand breaks, abasic sites and base damages (Demple & Harrison, 1994; Freidberg et al., 1995).

The discovery in 1960s and 1970s that inflammatory phagocytes produce large quantities of oxygen radical superoxide anions as well as other non-radical ROS, provided a basis for a plausible and testable hypothesis by which inflammation and carcinogenesis might be related i.e. that ROS generated by inflammatory phagocytes can cause injury to target cells which contributes to cancer development (Weitzman & Stossel, 1981). Activated human neutrophils were able to produce DNA strand breaks in murine erythroleukemia cells (Birnboim, 1983). Studies have shown that DNA damage caused by activated phagocytes was much more complex than caused by exogenously added H$_2$O$_2$ (Shacter et al., 1988). PMA stimulated phagocytes damage their own DNA in the process (Birnboim, 1982; Birnboim & Kanabuskaminska, 1984). In addition to strand breaks, activated human neutrophils and macrophages have shown to produce mutations in bacteria and many mammalian cells (Farr et al., 1986; Nassi-Calo et al., 1989). Most of the mutations observed were point mutations, either base substitution or base deletion. Activated phagocytes from normal donor produced sister chromatid exchange in Chinese hamster ovary (CHO) cells but not the phagocytes from patients with chronic granulomatous disease (Weitberg et al., 1983). It has been shown that phorbol-activated phagocytes could oxidise bleomycin A2 and benzo[a]pyrene to the derivatives that are mutagenic, covalently bind to DNA, and cause sister chromatid exchanges (Trush et al., 1986). Oxidative DNA damage is predominantly repaired by base excision repair proteins. These distinct glycosylases recognise specific oxidative lesions and cleave the N-glycosidic bond, releasing damaged base. The resulting abasic site can then be removed by an apurinic/apyrimidic endonuclease. These repair proteins are themselves potentially vulnerable to oxidative damage by NO because of their active site sulphydryl (Starke et al., 1997), tyrosine side chains. Some DNA repair enzymes like O$^\circ$-alkylguanine DNA alkyl transferase (Laval & Wink, 1994) and formamido pyrimidine-DNA glycosylase (Wink & Laval, 1994; O’conner et al., 1993), T$_4$ DNA ligases (Lindhal & Barnes, 1992) and poly (ADP) ribose polymerase have zinc finger motifs in
their active sites that may also be inactivated by NO nitrosylation of thiol moieties of their cysteine residues. It therefore appears that the integrity of the cell may be challenged during exposure to high concentrations of NO not only by direct oxidative damage to DNA but also by potential NO mediated disruption of DNA repair enzymes.

Other studies have indicated that when compared with nuclear DNA, mitochondrial DNA (mtDNA) contains an elevated basal level of base damage such as 8-oxoguanine (Richter et al., 1988). Oxidative mtDNA damage has been linked to the onset of specific human diseases such as neuronal degeneration, cardiovascular disease and aging (Ames et al., 1993; Trischler & Medori, 1993; Shigenaga et al., 1994; Wallace et al., 1995). The increased susceptibility of the mitochondrial genome to DNA damage could be due to several factors (i) the absence of complex chromatin organisation, which may serve as a protective barrier against ROS, (ii) alterations in DNA repair activity, (iii) the presence of localised metal ions that may function as catalysts in the generation of ROS and (iv) the stimulation of secondary ROS reactions due to damage to the electron transport chain and/or through lipid peroxidation. One potential source of continued DNA damage is through the generation of secondary ROS, such as lipid peroxidation products. Lipids within the inner mitochondrial membrane contain components of the electron transport chain, many of which contain transition metal ions. Stimulation of both radical and non-radical species through metal catalysed lipid peroxidation reactions have been shown to damage DNA (Vaca et al., 1988; Hruazkewycz, 1992; Stohs & Bagchi, 1995; Zastawny et al., 1995). Proximity of mtDNA to inner mitochondrial membrane is responsible for higher extent of damage to mtDNA. As 1-2% of all oxygen consumed with in the cell is thought to be released from mitochondria as ROS (Chance et al., 1979), it might be expected that ROS mediated damage to mitochondria may inactivate electron transport complexes or inhibit mtDNA transcription, thereby altering normal mitochondrial functions.

**Biological Damages by Nitrosative Stress**

Chemical reactions of NO can be divided into direct and indirect effects. For the direct effects, NO reacts directly with biological target, e.g. with heme
moiety of soluble guanylyl cyclase. For these effects, NO is released at low concentrations (<1 μM) and reaction of NO with heme of guanylyl cyclase is extremely fast (Kharitonov, 1997). The combination of low concentration of NO and fast reaction kinetics is the hallmark of effects of NO at physiological conditions. Indirect effects of NO occur during pathophysiological and toxicological events. In such conditions NO initially reacts with O₂⁻ or O₂, that form intermediate reactive nitrogen dioxide species, prior to the reaction with final target (Wink & Mitchell, 1998). These RNI can alter a wide range of biological macromolecules such as proteins, lipids and DNA and are thought to play a pivotal role in NO mediated cell death. Generally, in indirect effects, local concentration of NO is high which is sustained for prolonged periods of time. These attributes characterise NO production catalysed by inducible enzyme iNOS, suggesting that most indirect effects are likely to occur in the vicinity of activated macrophages or other cells expressing iNOS after immunostimulation and in disease states. In the presence of O₂⁻, NO enhances lipid peroxidation by ONOO⁻ mediated and metal independent mechanism. NO alone serves as a potent terminator of radical chain propagation reactions and can yield novel nitrogen-containing lipid oxidation products (Rubbo et al., 1994). NO by virtue of its lipophilicity and high reactivity for lipid peroxy radical species also prevents vitamin E depletion during lipid peroxidation. It has been shown that reaction between NO and chain propagating peroxy radical species is 3-times faster than for vitamin E (O’Donnell et al., 1997). It has been shown that NO exhibits antioxidant effect against metal catalysed lipid oxidation (Rubbo et al., 1996). Nitrosylation of cellular heme by NO prevented the increase of catalytic iron in concert with the inhibition of heme induced heme oxygenase and ferritin biosynthesis. It was suggested that NO acted as a buffer against excess heme by reducing its redox activity and slowing the release of free iron (Juckett et al., 1998). A principal fate of NO in vasculature is its reaction with hemoglobin. NO binds very rapidly to deoxyhemoglobin, forming a stable hemoglobin (Fe⁺⁺)NO complex. NO radical is usually first oxidised to generate species which can serve as a donor/source of nitrosonium ion (NO⁺). Free NO⁺ is never formed. NO⁺ is exceedingly reactive in biological systems and may only exist in gas
In interstellar space, the nitrosation of nucleophiles such as thiols, alcohols, and amines occurs via the generation of nitrosonium donor species (Williams, 1988; Wink et al., 1994; Simon et al., 1996). The most common NO+ donor in biological systems is N2O3 (Wink & Mitchell, 1998). All other donor species can be derived from N2O3 (Lewis et al., 1995). N2O3 is formed by the reaction between NO and O2 (Figure 2.3). The rate equation for NO/O2 reaction has second order dependency on NO and first order dependency on O2, indicating that life-time of NO is inversely proportional to its concentration (Ford et al., 1993). The second order dependency of NO/O2 reaction has several implications for biological systems. Only cells capable of generating a high flux of NO (e.g., iNOS catalysed synthesis) have the potential for causing nitrosative stress.

Excess NO formation may cause inappropriate vasodilation which is a key feature in septic organ failure (Stewart et al., 1995; Ullrich et al., 1999).
Extensive production of NO has been assumed to be a key event in the pathogenesis of several inflammatory diseases such as sepsis induced lung injury and asthma (Hamid et al., 1993; Kristoff et al., 1998; Hinder et al., 1999). NO produced in infected and inflammed tissues has been postulated to contribute to epithelial cell carcinogenesis by causing damage to DNA and proteins (Filep et al., 1997; Jaiswal et al., 2000). Indeed NO can directly oxidise DNA, resulting in mutagenic changes (Wink et al., 1991). NO has also been shown to inactivate DNA repair enzymes, hence contributes to accumulation of DNA damage (Jaiswal et al., 2000).

**Antioxidant Defense System**

The integrity and existence of an organism depends on proper homeostatic regulation. So the survival of the cell requires maintenance of cellular homeostasis. The evolutionary survival process has provided aerobic organisms with well balanced mechanisms to neutralise to oxidative effects of oxygen and its reactive metabolites. These self-sustained protective components are classified as "antioxidant defense system" (Davies, 1986; Heffner & Repine, 1989). Such a system encompasses many substances (Cutler, 1984), which are also known as antioxidants, free radical scavengers, chain terminators or reductants. Chemically, an antioxidant is a compound that significantly inhibits or delays oxidation, when present at low concentrations as compared to that of an oxidisable substrate (Halliwell, 1991). Krinsky (1992) has proposed a much broader and practical definition of antioxidants as compounds that protect biological systems against potentially harmful effects or processes/reactions that can cause excessive oxidations. Antioxidants can be divided into intracellular [glutathione peroxidase, glutathione reductase (GpX, GR)] and extracellular (vitamin E,C), enzymatic [superoxide dismutase (SOD), catalase] and non enzymatic (GSH, ubiquinol), exogenous (carotene, flavonoids) and endogenous (GSH, uric acid). The antioxidant defense systems against oxidative stress are as diversified as the free radicals themselves. This variety, of substances is capable of scavenging many species of free radicals to provide maximum protection. These scavengers are strategically compartmentalised in subcellular organelles within the cell to provide maximum protection. For example, SOD,
catalase and Gpx are not only located in cytosol but are also localised in mitochondria where most of the intracellular free radicals are generated (Ji et al. 1988).

The most efficient way to eliminate undesirable toxic species is by means of catalysis. Families of antioxidant enzymes have evolved including superoxide dismutase (SOD), catalase, Gpx, GR etc. Superoxide dismutase, is often called primary defense, as it catalyses the dismutation of O$_2^-$ to H$_2$O$_2$. Its catalytic function was discovered by McCord & Fridovich, 1969. It exists in virtually all aerobic organisms. The rate of dismutation by SOD is $10^4$ times faster than chemical dismutation. By rapidly removing O$_2^-$, SOD reduces the production of OH$^-$ and ONOO$^-$ radicals via reactions:

\[
2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2
\]

SOD are classified into three distinct classes depending on their metal content Cu/Zn SOD, MnSOD, FeSOD. Although some of the SOD activity appears to be extracellular, the bulk of the activity is localised intracellularly, divided between mitochondria (MnSOD) and cytosol (Cu/ZnSOD) (McCord & Marecki, 1997). CuZnSOD and FeSOD constitute 85–90% of total tissue SOD activity while MnSOD constitutes 10–15 % of the total SOD activity. In addition to superoxide dismutase activity, CuZnSOD also has peroxidase activity. Unlike MnSOD, CuZn SOD is inactivated by its product H$_2$O$_2$ (Yim et al, 1990). MnSOD is an inducible enzyme. Induction of MnSOD has been demonstrated to irradiation (Oberly et al, 1987), hyperoxia (Housset & Juno, 1982), paraquat (Krall et al. 1988) and LPS (Shiki et al, 1989). Biological importance of SOD has been demonstrated in simple organisms. E. coli deficient in SOD shows increased rate of mutagenesis (Touoti & Farr, 1990). Such mutant E. coli, showed more susceptibility to oxidative stress and develop greater resistance to oxidative challenge following reintroduction of SOD gene (Harris, 1992).
Catalase is a major primary antioxidant defense component that primarily works to catalyse the decomposition of H$_2$O$_2$ to H$_2$O (Cheng et al., 1981), sharing this function with glutathione peroxidase (GpX). The two enzymes have different substrate affinities. In the presence of low H$_2$O$_2$ levels, organic peroxides are preferentially catalysed by peroxidase, however at high H$_2$O$_2$ concentrations, they are metabolised by catalase. Tissue distribution of catalase is wide spread. The level of activity varies within the cell itself. The liver, kidney and red blood cells possess relatively high levels of catalase. In hepatocytes, peroxisomes exhibit exceedingly high catalase activity, but also found in microsomes and cytosol. (Thomas & Aust, 1985). GpX catalyses the reduction of H$_2$O$_2$ and organic hydroperoxides as follows:

\[
\text{GpX} \\
2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{} \text{GSSG} + 2\text{H}_2\text{O} \\
\text{or} \\
2\text{GSH} + \text{ROOH} \xrightarrow{} \text{GSSG} + \text{H}_2\text{O} + \text{ROH}
\]

Both selenium dependent and selenium independent types of GpX catalyse above reactions thus protecting against radical damage by reducing peroxides. Selenium dependent peroxidase is found in cytosol and exhibits a low capacity for H$_2$O$_2$ reduction. The selenium independent peroxidase utilises organic hydroperoxides as preferred substrates over H$_2$O$_2$.

Unlike catalase, GpX uses GSH for detoxification. GpX is more important than catalase in its function as GpX is located in the same cellular organelle as SOD (Badway & Karnovsky, 1980). Reduced glutathione is oxidised (GSSG) in GpX catalysed reaction. Glutathione reductase, a flavoprotein (FAD-containing enzyme), regenerates GSH from GSSG, at the cost of NADPH (Chance et al., 1979).

Reduced glutathione (GSH), a tripeptide, γ-glutamyl-cysteine glycine, is the most abundant nonenzymatic low molecular weight antioxidant (LMWA), virtually present in all mammalian cell systems. Reduced glutathione is characterised by its reactive thiol group and its γ-glutamyl bond which makes it resistant to peptidase attack. It serves as a nucleophile and effective reductant.
by interacting with numerous electrophilic and oxidising compounds such as 
H$_2$O$_2$, O$_2^-$, OH. GSH plays an important role in a variety of detoxification 
processes. GSH depletion increases the susceptibility of animals against 
cytotoxicity and affect drug interventions in neoplastic diseases (Al-Turk et al., 
1987). GSH readily reacts with free radicals, notably OH$^-$ and carbon radicals, 
by donating a hydrogen atom. Such reactions provide protection against OH$^-$, 
which is a major source of free radical damage.

The levels of GSH fluctuate under various physiological conditions 
including aging (Al-Turk & Stohs, 1981; Davies, 1986) and some neoplastic 
diseases (Farooqui et al., 1987). Biosynthesis of GSH from its constituent amino 
acids is catalysed by γ-glutamyl transferase and GSH synthase. Because γ-
glutamyl transferase is located in the plasma membrane with its active site facing 
the extracellular space, the increased demand for GSH would be expected to 
arise when increased peroxides are seen. Although much of the biochemical 
information available on GSH concerns the cellular contents, but GSH is also 
present in extracellular fluids. These extracellular pools of GSH function in 
detoxification and protection of cells from chemical and oxidant induced injuries. 
The intracellular pools typically contain 1-10 mM of GSH, and the extracellular 
pools of GSH are often 1-10μM range, with the exception in bile and alveolar 
lining fluid, where GSH concentrations were estimated in excess of 200-800 μM 
(Smith et al., 1996).

**Anti-inflammatory Drugs**

Various agents are used to treat pain and inflammation associated with 
various inflammatory conditions, but non-steroidal anti-inflammatory drugs 
(NSAIDs) are particularly effective in reducing pain where inflammation has 
sensitisation of pain receptors to otherwise painless chemical and 
mechanical stimuli. NSAIDs are among the most commonly and widely 
prescribed and used classes of drugs. Worldwide sales of NSAIDs amount to 
more than $6 billion/year. NSAIDs are used for the treatment of pain and 
inflammation in many conditions including osteoarthritis (OA) and rheumatoid 
arthritis (RA). Their principal mechanism of action is via inhibition of 
cyclooxygenases (COX), key enzymes in arachidonic acid metabolism.
(1971). The COX enzyme catalyses the initial steps in the conversion of arachidonic acid to various eicosanoids including prostaglandins (PGs) and thromboxanes. PGs play an important role in inflammation and their synthesis enhances during inflammatory responses via COX-catalysed arachidonic acid metabolism.

Cytokines such as IL-1, TNF-α and bradykinin are particularly important in eliciting pain of inflammation. These agents liberate prostaglandins and other mediators that promote hyperalgesia. Large doses of PGE2 & PGF2α cause intensive local pain. In general, NSAIDs do not affect the hyperalgesia or pain caused by direct actions of PGs. The analgesic effects of these drugs are due to inhibition of PG synthesis. However, some data suggest that analgesic effects of NSAIDs may also occur via mechanisms other than PG synthesis inhibition, which include antinociceptive effects at peripheral or central neurons (Gebhart & McCormack, 1994; Konttinen et al., 1994).

Regulation of body temperature requires a delicate balance between the production and loss of heat. The hypothalamus regulates the set point at which body temperature is maintained. In fever, this set point is elevated and NSAIDs promote its return to normal. Fever may be a result of infection, tissue damage, inflammation, graft rejection, malignancy or other disease states. A common feature in all these states is the enhanced production of cytokines such as IL-1β, IL-6, IFN-α, IFN-β, TNF-α. The cytokines increase the synthesis of PGE2 in circumventricular organs and near to the preoptic hypothalamic area. PGE2, via cyclic AMP, triggers the hypothalamus to elevate body temperature by promoting increase in heat generation and decrease in heat loss. NSAIDs suppress the response by inhibiting PGE2 synthesis. NSAIDs do not inhibit fever caused by directly administered PGs, but they do inhibit fever caused by agents that enhance the formation of cytokines, which presumably cause fever at least in part, by inducing the endogenous synthesis of PGs.

Another proposed mechanism for the effects of NSAIDs is via inhibiting expression or activity of some cell adhesion molecules, which are essential for the onset of inflammatory response. Such effects have been described for some NSAIDs and not others, suggesting that interference with the action of cell
adhesion molecules is not a common mechanism for all NSAIDs (Diaz-Gonzalez & Sanchez-Madrid, 1998). Nonetheless, effects on cell adhesion molecules may contribute in part to the anti-inflammatory actions of some NSAIDs. Novel classes of anti-inflammatory drugs directed against cell adhesion molecules are under active development (Kavanaugh et al., 1994; Rao et al., 1994; Endermann et al., 1997).

Apart from their well-known, antipyretic, analgesic and anti-inflammatory effects, they have also been used in the treatment of familial adenomatous polyposis (Giardiello et al., 1993). Epidemiological studies indicate that intake of NSAIDs has been inversely correlated with fatal cancers of oesophagus, stomach, colon & rectum (Isomaki et al., 1973; Marnett, 1992; Thun et al., 1993; Gridley et al., 1993; Giovannucci et al., 1994). NSAIDs have been shown to inhibit the growth of cancer cell lines and induce apoptosis in vitro, which was considered to be an important mechanism in their antineoplastic activity (Hanif et al., 1996; Zhu et al., 1999). Despite their demonstrated efficacy in reducing pain & inflammation and their antineoplastic properties, NSAIDs use has also been associated with adverse side effects, including peptic ulcer disease, gastric bleeding, intestinal perforation and decreased kidney functions (Soll et al., 1991; Eberhart & DuBois, 1995; Smith & Dewitt, 1995).

Less than a decade ago, it was demonstrated that the COX enzymes exist in two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most of the tissues and governs the production of AA metabolites that are necessary for the maintenance of physiologic homeostasis. In contrast, COX-2 is not detectable in most normal tissues but is induced by cytokines, growth factors, oncogenes, serum and tumour promoters (Kujubu et al., 1991; O'Banion et al., 1991; Jones et al., 1993; DuBois et al., 1994; Rimarachin et al., 1994; Xie & Herschman, 1995). The two isoforms of COX are about 60% homologous within a species. Moreover, all amino acids identified as important for catalysis by COX-1, are conserved in COX-2. There are significant differences between the gene and promoter structures of COX-1 and COX-2, their mRNA stability and intracellular localisation of gene products. COX-1 gene, which is 22 kb in length, contains 11 exons and 10 introns, whereas, COX-2 is approximately 8 kb and
Review of Literature
contains 10 exons and 9 introns (Fletcher et al., 1992; Xie et al., 1993; Williams & Dubois, 1996). The exons of two genes are similar in size, but the introns of COX-2 gene are considerably smaller than those of COX-1 gene. The more instability of COX-2 mRNA as compared to COX-1 has been attributed to the presence of 17 copies of Shaw-kamen sequence (AUUUA) in 3′-UTR of COX-2 (Shaw & Kamen, 1986; Kosaka et al., 1994). These instability sequences are present in many immediate early response genes. The 5′-flanking region of COX-2 contains a TATA box, numerous cis-acting promoter elements including NFκB, NF-IL-6, CRE sites. Based on its cDNA sequences, the predicted molecular weight of COX-1 is 65.5 kDa, excluding the signal peptide. However, COX-1 migrates as a single band on SDS-PAGE with an Mr of about 72 kDa. This difference is predicted and observed values of Mr is a consequence of post-transcriptional addition of three N-linked oligosaccharides at Asn-67, 143 and 409 (Otto et al., 1993). In contrast to COX-1, COX-2 usually appears as a doublet on SDS-PAGE with M_r of 72 and 74 kDa. The 72 kDa species contains 3-N-linked oligosaccharides. The 74 kDa species contains an additional 4th N-linked oligosaccharide at Asn580. This site is only partially glycosylated, yielding two subpopulations of COX-2 molecules that move slightly differently on gel (Otto et al., 1993). The carboxy terminal of COX-2 contains a unique 18-amino acid region that is absent in COX-1. Antibodies raised against this unique carboxy terminal peptide region are used to distinguish COX-2 from COX-1 (Habib et al., 1993).

COX-1 and COX-2, both are membrane bound, but have different patterns of subcellular localisation. COX-1 is primarily present on endoplasmic reticulum (ER), COX-2 is located in both ER and perinuclear envelope (Morita et al., 1995). Possibly, PGs produced by different isozymes may be compartmentalised, with the COX-2 products having effects in nucleus (e.g. regulation of target gene expression). Data also support the fact that COX-1 and COX-2 utilise different intracellular pools of arachidonic acid (Chulada et al., 1996) with different functional consequences.

Prostaglandins, in addition to their housekeeping homeostatic functions, also appear to be important in the pathogenesis of cancer because they affect
mitogenesis, cellular adhesion, immune surveillance and apoptosis (Sheng et al., 1998). Moreover, formation of PGs increases in cancerous tissues as compared to normal tissue from which cancer has arisen (Jung et al., 1985; Vanderveen et al., 1986; Bennett et al., 1987; Rigas et al., 1993; Lupulescu, 1996). The increased amounts of PGs in tumours reflect enhanced synthesis, which occurs by COX-catalysed metabolism of arachidonic acid. The expression of COX-2 at mRNA and protein levels, but not of COX-1, has been found to be elevated in epithelial cells within human colorectal, oesophageal, head and neck and lung cancers (Eberhart et al., 1994; Huang et al., 1998; Hida et al., 1998; Wolff et al., 1998; Zimmerman et al., 1999; Chan et al., 1999). COX-2 is inducible in endothelial cells, monocytes, macrophages in response to pro-inflammatory cytokines such as TNF-α and IL-1 (Herschman, 1996). NSAIDs being COX-inhibitors have been found to reduce the incidence of colon cancer, which has been inversely associated with the chronic intake of NSAIDs (Rao et al., 1995; Jacoby et al., 1996; Reddy et al., 1996; Schiff & Rigas 1997; Langman & Boyle, 1998; Reddy et al., 2000). In addition to colon carcinogenesis, evidence is available for the possible effects of NSAIDs against the development of cancers of mammary gland, skin, liver and urinary bladder (Klan et al., 1993; IARC, 1997; Denda et al., 1997; Okajima et al., 1997; Harris et al., 2000). COX-1 is essential for housekeeping functions whereas COX-2 expression is associated with inflammatory and cancerous conditions. Adverse effects associated with the use of NSAIDs are thought to be caused by non-selective inhibition of both COX-1 and COX-2 by conventional NSAIDs by impairing prostaglandin dependent mucosal protective mechanisms. COX-1 inhibition leads to reduction in bicarbonate secretion, mucosa formation and vascular actions (Hawkey, 1996). Other side effects, unrelated to PG synthesis include a topical action on the gastric mucosa (Mahmud et al., 1996).

In order to avoid these adverse effects associated with intake of NSAIDs, the small differences between the NSAID binding sites of COX-1 and COX-2 have been exploited in developing selective inhibitors of COX-2 (Marnett et al., 1999). The single amino acid difference of isoleucine by valine in the NSAID binding site of COX-2, results in an additional pocket off to the site of central
active site of COX-2. Compounds designed to bind to this additional space are potent and selective inhibitors of COX-2. This discovery led to the development of specific inhibitors, which are 1.25 to 1000 times more selective for COX-2 than COX-1. According to the COX-2 and COX-1 selectivity hypothesis, it would be expected that these drugs have lesser effects on physiological prostaglandins production (by sparing COX-1 activity) than non-selective NSAIDs, while preserving the ability to inhibit the PG production in inflammation, hence expected to have an improved gastrointestinal profile, with no loss of efficacy as anti-inflammatory agents (Lucina & Hawkey, 2000). Clinical trials in patients with rheumatoid arthritis and dental pain have demonstrated that COX-2 specific inhibitors are therapeutically as effective as conventional NSAIDs (Saag et al., 1998; Ehrich et al., 1998; Simon et al., 1998). Endoscopic studies have shown a significant reduction in gastric ulcers and mucosal damage compared with conventional agents (Simon et al., 2000). Moreover, COX-2 specific inhibitors have no measurable effects on platelet aggregation and they do not prolong bleeding time (Simon et al., 1998). In the year 2000, a panel of experts convened by the international COX-2 study group, provided recommendations for the use of COX-2 specific inhibitors in clinical practice (Lipsky et al., 2000). Evidences have been provided for COX-2 specific inhibitors, like nimesulide, meloxicam, coxib family members that they are clearly safer for the gastrointestinal tract than traditional NSAIDs. They are associated with the development of fewer ulcers and adverse effects related to gastrointestinal tract (Buttereit et al., 2001).

Nimesulide

Nimesulide (CAS-51803-78-2), 4-nitro-2-phenoxyethane sulphonanilide, is weakly acidic, non-steroidal anti-inflammatory drug having analgesic and antipyretic effects. Nimesulide is one of the most commonly prescribed and consumed agents for a variety of inflammatory and pain states, administered orally or rectally, twice daily (Davis & Brogden, 1994). It is effective in reducing pain associated with osteoarthritis, cancer, thrombophlebitis, oral surgery, and dysmenorrhea in adults, reducing pain associated with general surgery in adults and children and pain, fever and inflammation accompanying respiratory
tract infections, otorhinolaryngological diseases and traumatic injury in adults and children. Nimesulide has been well tolerated by adults, elderly and paediatric patients in clinical trials and large postmarketing surveillance studies.

![Structure of Nimesulide](image)

**Figure 2.4: Structure of Nimesulide**

Nimesulide has been found to be a selective COX-2 inhibitor *in vivo* in human beings (Cullen et al., 1998), consistent with the finding that nimesulide is associated with a very low incidence of adverse side effects especially in gastrointestinal tract (Rainsford, 1999; Bjarnason & Thodleifsson, 1999). In various experimental models, nimesulide exhibited protective action towards colon carcinogenesis (Fukutake et al., 1998; Tardieu et al., 2000), formation of intestinal polyps (Nakatsugi et al., 1997), urinary bladder carcinogenesis induced by N-butyl-N (4-hydroxybutyl) nitrosamine (Kitayama et al., 1999) and against 2-amino-1-methyl-6-phenyl imidazo [4,5-b] pyridine (PhIP) induced mammary carcinogenesis in female Sprague-Dawley rats (Nakatsugi et al., 2000). In *in vitro* studies, nimesulide inhibits proliferation of non small cell lung cancer cells via inducing apoptosis and reduced IC$_{50}$ of various anticancer agents effectively (Hida et al., 2000). Worldwide experience with nimesulide in comparison to other NSAIDs such as diclofenac, naproxen, etodolac, confirms that the drug demonstrates superior gastrointestinal tolerability and low toxicity to kidneys, hence, is a good choice for the long term treatment of diseases like osteoarthritis (Bennett & Villa, 2000; Huskisson, 2001). COX-2 specific agents are desirable not only because they spare COX-1, and avoid gastrointestinal toxicity but also because they are weakly acidic, and therefore avoid substantial accumulation in gastric mucosa. The short term endoscopic studies indicate that mucosal injury increases with the amount of NSAIDs. More acidic NSAIDs tend to cause greater gastric damage. Conventional acidic NSAIDs increase the permeability of human
small intestine, probably by a non-prostaglandin mechanism but nimesulide does not do so, probably because of its very weak acidic nature (Bjarnason & Thjodleifsson, 1999).

Nimesulide seems to act via various mechanisms other than COX-2 inhibition. These include (a) reduction in cytokine action/release, (b) reduction in histamine release, (c) reduction in release of enzymes that degrade cartilage and (d) inhibiting neutrophil actions and reducing release of superoxide anions and other toxic substances from neutrophils (Rainsford, 1999; Bennett & Villa, 2000). Nimesulide has been shown to inhibit the TNF-α and IL-1β stimulated production of PGs (Sato et al., 2002). Cytokines like TNF-α, IL-1β, INF-γ and PMA induce COX-2 activity leading to varying amounts of PGE2 (Hulkower et al., 1997; Pang & Knox, 1997). Nimesulide and other NSAIDs inhibit LPS induced TNF-α levels in vitro as well as in vivo in some experimental models (Azab et al., 1998; Karmeli et al., 2000; Shemi et al., 2000; Azab & Kaplanski, 2001). Nimesulide inhibits the neutrophil adhesion and migration in TNF-α activated endothelial cells (Dapino et al., 1994). COX-2 expression at mRNA and protein has been inhibited by nimesulide and this activity has been proposed to be at least in part via inhibition in cytokine mediated generation of free radicals (Fahmi et al., 2001), but was unable to inhibit the cytokine mediated NFκB activation (Fahmi et al., 2001). Nimesulide inhibits oxidative stress by suppressing the release of O₂⁻ anions in colonic inflammation (Tardieu et al., 2000) and by decreasing O₂⁻ production in PMA and fMCP stimulated neutrophils (Bevilacqua et al., 1994). Earlier studies regarding the effects of nimesulide on chemotaxis, phagocytosis and oxidant production are on neutrophils and mostly in vitro (Dallegri et al., 1990; Ottonello et al., 1992; Capecchi et al., 1993). But studies are lacking about the antioxidant effects of nimesulide on the functions of alveolar macrophages under lung inflammatory conditions. Moreover, its modes of action as an anticarcinogenic agent are poorly understood at cellular and molecular levels.