INTRODUCTION
Tyrosine is an aromatic amino acid, containing a phenyl ring attached to a methylene (-CH$_2$-) group (Fig. 1). The aromatic ring contains a hydroxyl group, which makes tyrosine less hydrophobic and more reactive than other amino acids. It is a non-essential amino acid, which is produced in cells by hydroxylating the essential amino acid, phenylalanine. Half of phenylalanine required by the body goes into the production of tyrosine, if the diet is rich in tyrosine itself, then the requirement for phenylalanine is reduced by about 50%. Phenylalanine hydroxylase is a mixed-function oxygenase: one atom of oxygen is incorporated into water and the other into the hydroxyl group of tyrosine. The reductant is the tetrahydrofolate related cofactor tetrahydrobiopterin, which is maintained in the reduced state by the NADH-dependent enzyme dihydropteridine reductase (Fig. 2).

Tyrosine is a neutral, genetically coded amino acid and is important to the structure of almost all proteins in the body. It is also a precursor of several neurotransmitters like dopa, dopamine, norepinephrine and epinephrine. Through its effect on neurotransmitters, it may affect several health conditions including Parkinson’s disease, depression and other disorders. Because of its role as a precursor of norepinephrine and epinephrine (two of body’s main stress related hormones), it may also ease the adverse effects of environmental, psychosocial and physical stress. Thyroid hormones, which have a role in almost every process in the body, also contain tyrosine as part of their structure. Tyrosine also aids in the function of the adrenal and pituitary glands. Because tyrosine binds unstable molecules (called free radicals) that can potentially cause damage to the cells and tissues, it is considered a mild antioxidant. Thus, tyrosine may be useful for people who have been exposed to harmful chemicals (such as from smoking) and radiation.
Fig. 1. 3-D Structure of tyrosine
Fig. 2. Biosynthesis of tyrosine from phenylalanine
**Poly L-Tyrosine**

Poly L-tyrosine is a homopolymer of L-tyrosine, i.e., many tyrosine residues joined by peptide bonds to form a polypeptide chain, which is unbranched. The mean molecular weight of an amino acid is 110 Dalton, and so the molecular weights of most polypeptides chains are between 5500 and 220,000 Daltons. The polymer of tyrosine is found to precipitate out of solution at pH 10.3. Fasman et al. (1964) reported that the apparent pKa of poly L-tyrosine in 0.2 M NaCl solution is approximately 11.5. Poly L-tyrosine has been the object of several studies due to its conformational dependence on the properties of various media. In particular, it has been found that poly L-tyrosine undergoes a solvent induced structural transition in going from one solution to another (Yasui and Keiderling, 1986). For this reason, the establishment of the conformation in which aromatic poly amino acids exist in solution is difficult. This explains the various structure proposals in the case of Poly L-tyrosine (Beychok and Fasman, 1964; Fasman et al., 1964; Applequist and Mehr, 1966; Patrone et al., 1970; Quadrifoglio et al., 1970; Jurgen, 1971). For example, poly L-tyrosine can exist in either a helical or a random-chain conformation in the un-ionized form. The optical rotatory dispersion (ORD) of helical poly L-tyrosine (in 0.2 M NaCl, pH 11.2) has been recorded suggesting that tyrosyl-tyrosyl interactions occur in the helical conformation and poly L-tyrosine is a right-handed helix (Fasman et al., 1964), whereas it has previously been shown that poly L-tyrosine forms an intramolecular antiparallel β-structure in aqueous solution (Auer and Miller-Auer, 1986).

There is a correlation between the size of a macromolecule and its immunogenicity. The best immunogens tend to have a molecular mass approaching 10 kDa. Generally, substances with a molecular mass of less than 5-10 kDa are poor immunogens. Size is not by itself sufficient to make a molecule immunogenic, other properties are needed. Synthetic homopolymers tend to lack immunogenicity regardless of their size whereas for a synthetic copolymer e.g. of glutamic acid and lysine, requires a minimum molecular weight of 30-40 kDa. The addition of tyrosine to the copolymer reduces the required minimum molecular weight to 10-20 kDa. The addition of aromatic amino acids profoundly enhances the immunogenicity of these synthetic polymers. All four levels of
protein organization—primary, secondary, tertiary and quaternary contribute to the structural complexity of a protein and hence affect its immunogenecity (Goldsby et al., 2000, Kuby).

**Free Radicals**

A free radical may be defined as any chemical species that contain one or more unpaired electrons in the outer shell of the molecule. They are extremely reactive and have a very short half-life. They quickly react with other compounds, trying to capture the needed electron to gain stability. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction. Once the process is started it can cascade, finally resulting in the disruption of a living cell (Freeman, 1984; Halliwell and Gutteridge, 1984; Cadenas, 1989). Free radicals can be generated both *in vivo* and *in vitro* by one of the following mechanisms:

1. By the hemolytic cleavage of a covalent bond of a molecule, with each fragment having an unpaired electron (Von Sonntag, 1987).
2. By the loss of a single electron from a molecule.
3. By the addition of a single electron to a molecule.

The latter, electron transfer, is a more common process in biological synthesis than hemolytic fission, which generally requires high energy input from either high temperature, UV light or ionizing radiations. Free radicals can be negatively charged, positively charged or electrically neutral (Cheeseman and Slater, 1993). The most important free radicals in the body are derivatives of oxygen, commonly known as reactive oxygen species (ROS) and derivatives of nitrogen known as reactive nitrogen species (RNS) (Table 1).

In living cells, free radicals are produced continuously as by-products during normal aerobic metabolism (Halliwell and Aruoma, 1991) or deliberately as in phagocytosis (Cheeseman and Slater, 1993) as well as in pathologic conditions such as tissue ischaemia, cancer, inflammation and degenerative diseases (Halliwell and Gutterridge, 1984). However, environmental factors such as pollution, radiation, cigarette smoke and herbicides can also spawn free radicals. Table 2 lists some of the sources of oxidative stress.
TABLE 1
Examples of ROS and RNS

<table>
<thead>
<tr>
<th>REACTIVE OXYGEN SPECIES</th>
<th>REACTIVE NITROGEN SPECIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. O₂ in its triplet state (³O₂) or singlet O₂ (¹O₂)</td>
<td>1. Nitric oxide (NO⁻)</td>
</tr>
<tr>
<td>2. Superoxide anion (O₂⁻)</td>
<td>2. Peroxynitrite (ONOO⁻)</td>
</tr>
<tr>
<td>3. Hydroxyl radical (’OH)</td>
<td>3. Nitrate (NO₃⁻)</td>
</tr>
<tr>
<td>4. Hydrogen peroxide (H₂O₂)</td>
<td>4. Nitrite (NO₂⁻)</td>
</tr>
<tr>
<td>5. Hypochlorous acid (HOCl)</td>
<td>5. Nitrogen dioxide (NO₂⁻)</td>
</tr>
</tbody>
</table>
### TABLE 2

Source of Free Radicals in the Body

<table>
<thead>
<tr>
<th>Source</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mitochondrial electron transport</td>
<td>1. Leakage of superoxide due to inefficient reduction of $O_2$</td>
</tr>
<tr>
<td>2. Transition metal ions</td>
<td>2. Copper and iron facilitate hydroxyl radical formation</td>
</tr>
<tr>
<td>3. Inflammation</td>
<td>3. Free radicals released by activated phagocytes</td>
</tr>
<tr>
<td>4. Enzymes e.g. xanthine oxidase</td>
<td>4. Release superoxide during reperfusion of ischemic tissue</td>
</tr>
<tr>
<td>5. Drug metabolism</td>
<td>5. Free radical intermediate created during metabolism</td>
</tr>
<tr>
<td>7. Radiation</td>
<td>7. X ray, UV light</td>
</tr>
</tbody>
</table>
Reactive Nitrogen Species

More recently, the role of reactive nitrogen species, such as nitric oxide and its by-products such as peroxynitrite (ONOO\(^-\)), nitrate (NO\(_3^-\)) and nitrite (NO\(_2^-\)) have been shown to directly affect cellular signaling, vasodilation and immune response (Drew and Leeuwenburg, 2002).

Nitric oxide – A Biological Messenger

Since its publicized discovery in 1987, nitric oxide has become the focus of major research on its control of many cellular functions, including circulation of the blood, regulating activities in the brain, lungs, liver, kidneys, stomach, gut, genitals and other organs. The 1998 Nobel Prize in physiology or medicine recognized the biological significance of nitric oxide (Morgan, 2000).

Nitric oxide is an uncharged molecule with an unpaired electron. These characteristics of nitric oxide make it an ideal messenger molecule. Uncharged NO can diffuse freely across membranes. With an unpaired electron, it is called a radical molecule, which is highly reactive having a half life of 2-30 seconds and after transmitting a signal, it spontaneously decays into nitrite (Lowenstein and Snyder, 1994).

NO is considered to be an ubiquitous endogenous system which takes part in body's homeostatic regulations and in pathological events. Nitric oxide is formed by nitric oxide synthase (NOS) in an unusual reaction that converts arginine and oxygen into citrulline and nitric oxide (White and Marietta, 1992; McMillan et al., 1992; Mariotto et al., 2004). Three enzyme isoforms produce nitric oxide (Forstermann et al., 1990; Domenico, 2004): a neuronal nitric oxide synthase (NOS 1) and endothelial NOS (NOS 2 and NOS 3) (Fig. 3). The inducible form (NOS 2) is found in many cell types, including mononuclear phagocytes, hepatocytes and chondrocytes (Bredt et al., 1991a; Marsden et al., 1992). The constitutive isoforms (NOS 1 and NOS 3) release small amounts of nitric oxide for a brief period to signal adjacent cells, whereas the inducible isoform releases large amounts of nitric oxide continuously to eliminate bacteria and parasites. Nitric oxide diffuses out of the cell that generates it and into target cells, where it interacts with specific molecular targets (Table 3).
Fig. 3. The production of NO and L-citrulline from L-arginine, O₂, and NADPH-derived electrons.
### TABLE 3
Molecular Targets of Nitric Oxide

<table>
<thead>
<tr>
<th>Nitric oxide target molecules</th>
<th>Mechanism</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins</td>
<td>Binding to haem iron</td>
<td>Guanylate cyclase, Hemoglobin, NADPH-ubiquinone oxidoreductase, succinate-ubiquinone oxidoreductase</td>
</tr>
<tr>
<td></td>
<td>Binding to iron sulphur cluster</td>
<td>Cis-aconitase, ribonucleotide reductase</td>
</tr>
<tr>
<td></td>
<td>ADP ribosylation</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Nucleic acids</td>
<td>Deamination</td>
<td>DNA bases</td>
</tr>
<tr>
<td>NO radical molecule</td>
<td>Reacts with</td>
<td>Superoxide; hydroxyl radical to form peroxynitrite</td>
</tr>
</tbody>
</table>
NO in Cardiovascular and Pulmonary Systems

In 1980, Furchgott and Zawadski discovered that the sections of the aorta would relax in response to agonists only if the inner lining of endothelial cells were intact. However, aortic rings with no endothelial cells could not relax. Endothelial cells thus released an agent that relaxed vascular smooth muscle and this endothelial derived relaxation factor was found to be nitric oxide.

Nitric oxide automatically regulates blood flow in response to local changes in some regions of the vasculature (Wennmalm et al., 1990). It interacts with smooth muscle, causing relaxation of the blood vessels (Yoshiki, 1995). Nitric oxide also diffuses into the blood stream to produce NO-haemoglobin (Iwamoto et al., 1994) or met-haemoglobin. NOS have also been found inside platelets (Radomski et al., 1990a). It reduces clotting by inhibiting platelet aggregation (Radomski et al., 1990b; Pasqui et al., 1991; Varela et al., 1992; Vallance et al., 1992 and Ivanova et al, 1993) and adhesion (Radomski et al., 1987a; Sneddon and Vane, 1988; de Graaf et al., 1992 and Siney and Lewis, 1992). The molecular basis of this mechanism includes NO activating platelet guanylate cyclase and adenylate cyclase (Yamakado et al., 1982; Radomski et al., 1987b; Gryglewski et al., 1989; Maurice and Haslam, 1990; Bowen and Haslam, 1991; Nolte et al., 1991), inhibiting platelet phospholipase C (Durante et al., 1992) or attaching ADP-ribose to platelet glyceraldehyde-3-phosphate dehydrogenase (Brune et al., 1990). Sheer stress, an increase in blood flow through vessels, is another physical stimulus to which endothelial cells respond by increasing NO production (Buga et al., 1991; Kelm et al., 1991). A basal level of NO also regulates blood flow in the brain (Toda and Okamura, 1990; Tanaka et al., 1991; Faraci and Breese, 1993), heart (Chu et al., 1990; Lowenstein and Snyder, 1994), lung (Fineman et al., 1991), gastrointestinal tract (Iwata et al., 1992) and kidney (Baumann et al., 1992; Ribeiro et al., 1992; Yukimura et al., 1992; Deng and Baylis, 1993). Thus NO is an endogenous auto-regulator of blood flow.

NO as a Neurotransmitter

Nitric oxide is a simple molecule with diverse biological functions. NO and related reactive nitrogen oxide species (RNOS) mediate intricate physiological and
pathophysiological effects in the central nervous system. Depending on environmental conditions, NO and NOS can initiate and mediate neuroprotection or neurotoxicity either exclusively or synergistically with other effectors (Boje, 2004). It is counter intuitive to consider a toxic, highly reactive gas as a crucial neurotransmitter but abundant evidence indicates that NO is indeed a neurotransmitter in the peripheral and central nervous systems. It is generated in the neuron and the glial cell of these nervous systems. Immunohistochemical studies have localized NOS to discrete neuronal populations, such as those in the myenteric plexus throughout the gastrointestinal tract (Bredt et al., 1990; Young et al., 1992; Grozdanovic et al., 1994; Morgan, 2000). Neurotransmitters are normally stored as stable chemicals in the synaptic vesicles of nerve terminals. In contrast, NO does not rely on vesicle storage. NO is synthesized as needed whereupon it promptly diffuses in all directions into nearby neurons, by passing conventional neural receptors (Snyder and Bredt, 1992). The signaling action of NO is both intra- and intercellular (Huang et al., 1998). The radical may exhibit either a up or down regulatory mode in the spinal cord depending upon the types of fibers activated and the intensity of signal input (Callsen et al., 1999).

NO also directly regulates the formation of ion channels or receptors. It modulates the potassium channel to regulate the neuronal transmission (Bolotina et al., 1994; Cohen et al., 1994; Miyoshi, 1994). NO also modulates intestinal reflexes by direct action on intestinal neurons of the enteric nervous system (Yuan et al., 1995). In the peripheral nervous system, the main function of NO is vasoregulation. It may also play a role in regulation of insulin release from pancreatic beta cells (Toda et al., 1994). The renal blood flow is suggested to be regulated indirectly with NO through the attenuation of sympathetic neuronal activity in addition to the direct action (Sakuma et al., 1992; Togashi et al., 1992). In presence of NO, weak input signals that might otherwise be unnoticed by the cell can undergo amplification and result in significant physiological responses (Peunova and Enikoiopov, 1993).

In addition to NO’s role in neurotransmission, it is also involved in neural development, neural regeneration and regulation of genetic expression (Yun et al., 1997). Evidence to support the hypothesis that overproduction of nitric oxide might be involved
in conditions such as cerebral ischemia, epilepsy and cerebral infarction is still controversial (Dawson et al., 1991; Mollace et al., 1991; Nowicki et al., 1991). Microglial cells can express the inducible form of NOS (Zielasek et al., 1992) and these cells have been implicated in the pathogenesis of multiple sclerosis, Alzheimer’s disease, Parkinson’s disease and dementia of the acquired immunodeficiency syndrome. Nitric oxide appears to elicit neurotoxicity by activating poly adenosine 5’-diphosphoribose synthase (PARS) (Zhang et al., 1994).

**NO in the Immune System**

The role of NO in the immune system is unique because NO is nonspecific and damages any cell or pathogen. In contrast, antibodies or cytotoxic T lymphocytes act by first recognizing specific pathogens or infected cells and then destroying them (Lowenstein and Snyder, 1992). Immune cells, including activated macrophage, nucleophile, monocyte, and Kupffer cells can release a greater amount of NO than endothelium or nerve cells. The major function of the NO from iNOS is cytostatic and the cytotoxic effects on invading microorganisms or tumor cells (Croen, 1993; Farias-Eisner et al., 1994). The mechanism of the cytotoxicity is in the following two categories. First is the inhibition of the respiration of mitochondria (Stadler et al., 1991). NO strongly interacts with and inhibits some enzymes in the electron transfer system, because such enzymes contain an iron-sulfur center in the catalytic site. Second is the direct modulation to the DNA synthesis to inhibit some enzymes (Garg and Hassid, 1989; Maragos et al., 1993). NO-releasing agents usually tend to inhibit cell proliferation and cell mitosis (Garg and Hassid, 1993), the expected effect is thymidine uptake. The radical is supposed to mediate the apoptosis of many cells (Albina et al., 1993). Although NO production by iNOS is essential for the defense system of an organism, it is sometimes related to pathological conditions, including sepsis, ischemia/reperfusion, acute pulmonary injury, multiple organ failure syndrome, and atherosclerosis. Septic shock is one of the most serious pathological conditions. It is still uncertain whether a complete scavenging of the NO is desirable for the improvement of the pathological condition (Yoshida et al., 1994).
Nitric Oxide, a Nuisance, at Worst a Poison

NO is a hydrophobic gaseous molecule that is highly diffusible and is highly reactive. It is a paramagnetic and diatomic molecule. It is also a free radical that has one pair electron in a 2p-π antibonding orbital, thus NO is extremely unstable and cannot maintain its original form for a very long time in a biological environment (Katayama, 1995). NO is a key participant in many physiological pathways in the body; however, its reactivity gives it the potential to cause considerable damage to the cells and tissues in its vicinity (Burney et al., 1997).

The reaction of NO with transition metals to form metal nitrosyls, is involved in both its regulatory and cytotoxic actions. The presence of porphyrin ligands markedly increases the affinity of NO for iron, and haem proteins are a major target (Gordge, 1998). The main trap for NO is oxyhemoglobin, which binds NO faster by five to six orders of magnitude than oxygen (Stamler et al., 1992). The reaction with haemoglobin produces nitrate and metahemoglobin (met-Hb) (Iwamoto et al., 1994). The basis of many biological actions of NO is the activation of guanylyl cyclase through binding to the haem prosthetic group of the enzyme (Moncada and Higgs, 1991). The enzyme increases the production of cGMP, modulating endothelium-dependent relaxation (Buga et al., 1989), platelet function (Radomski et al., 1990a,b) and nitricergic inhibitory transmission (Rand, 1992). Other NO-sensitive metalloproteins are NOS, cytochrome P450 (Bredt et al., 1991b), ferritin, ceruloplasmin, myoglobin, cyclo-oxygenase, catalase, ribonucleotide reductase and several components of the mitochondrial respiratory chain (Wink and Mitchell, 1998). These reactions have wide implications for the physiologic and toxic effects of NO. During sustained output of NO by inducible NOS, reaction with oxygen becomes significant, allowing the formation of dangerous intermediates such as N₂O₃. These can mediate oxidation and nitrosation of cysteine thiols and amino groups in both peptides and DNA bases, as well as nitration of tyrosine residues (Wink et al., 1994).

Reactions between radicals are among the fastest in nature and NO combines at almost diffusion limited rates with superoxide (O₂⁻⁻), peroxo (NOO⁻⁻) and hydroxyl radical (·OH). The product of NO and superoxide, peroxynitrite anion (ONOO⁻⁻), though
not itself a radical, is nevertheless a powerful oxidant and a cytotoxic mediator (Xie et al., 1996).

NO at submicromolar concentration competes with oxygen for cytochrome C oxidase to cause reversible inhibition of respiration (Brown, 1995). It can reversibly inhibit enzymes containing transition metals or free radical intermediates in their catalytic cycle (Beckman and Koppenol, 1996). NO in micromolar concentrations reversibly inhibits catalase and cytochrome P450 (Wink et al., 1993a). It can also inhibit ribonucleotide reductase, a critical enzyme for synthesis of DNA precursors that contain tyrosine radical (Kwon et al., 1991; Lepoivre et al., 1992). In rat kidney, proximal tubules subjected to hypoxia, increased NO generation which is associated with membrane injury, shown by cellular release of lactic dehydrogenase (Yaqoob et al., 1996). High output of NO generation can lead to inhibition of DNA synthesis and cytostasis (Gordge, 1998) and in some cases to P53 accumulation, DNA fragmentation and apoptosis. Cytotoxicity and DNA damage resulting from excessive production of NO in vivo, have the potential to trigger many diseases including various types of cancer. It is known that excess NO deaminates deoxyribonucleosides, deoxyribonucleotides, and intact DNA at physiological pH (Sugiura and Matsumoto, 1995). It also induces strand breaks and alkylation by nitrosamines formed by reaction of amines with nitrosating agents derived from NO (Ohshima and Bartsch, 1994; Tannenbaum et al., 1994; Liu and Hotchkiss, 1995; Wiseman and Halliwell, 1995).

Excessive NO production and increased iNOS induction in the liver have been reported in chronic liver diseases (Watanabe et al., 2001). NO stimulates angiogenesis and mediates the effect of different angiogenic molecules. In human tumors, NOS expression and activity correlate with tumor growth and aggressiveness through angiogenesis stimulation and regulation of angiogenic factor expression (Morbidelli et al., 2004). Overproduction of NO has also been implicated in the pathogenesis of SLE (Dixit and Ali., 2004) and chronic inflammatory bowel diseases. High concentrations of serum nitrite/nitrates and elevated urinary nitrate:creatinine ratios have been found in patients with rheumatoid arthritis (RA), osteoarthritis (OA), and in the spondylarthropathies (Morgan, 2000; Sandhu et al., 2003). Synovial fluid nitrite levels
are higher than those found in serum suggesting that NO may arise within the inflamed joint (Farrell et al., 1992; Ueki et al., 1996).

**Nitric Oxide and Apoptosis**

Living cells eventually die from either necrosis or apoptosis. In necrotic death, cells passively swell, mitochondria are disrupted, the cell membrane is lysed, and cellular components are released into surrounding tissues causing local edema and swelling.

The other form of cell death, apoptosis, is a process of controlled cell suicide whereby cells no longer needed by the body are eliminated. In apoptotic death an active process of cell shrinkage occurs, followed by phagocytosis preventing nearby tissue inflammation or damage (Nicotera et al., 1995). Controlled apoptosis is crucial to normal health. The process of embryonic growth and differentiation requires that surplus cells die and be removed. In later adult life, disrupted apoptosis allows development of neoplastic cells and certain autoimmune diseases. Excessive apoptosis, however, is likely involved in neurodegenerative diseases and in diabetes (Morgan, 2000).

NO has been termed as the 'mediator of lethal processes' from its close association with apoptosis. When the NO concentration reaches critical levels, determined by cell type and the local environment, regulated cell death occurs (Nicotera et al., 1995). NO causes oxidative stress. Mild oxidative stress leads to apoptosis whereas severe oxidative stress leads to excessive cellular damage and necrotic cell death. NO also affects mitochondria in three ways: by reversible inhibition of respiration; irreversible inactivation of mitochondrial enzymes; and induction of the mitochondrial permeability transition. It may also nitrosylate critical thiol residues on creatine kinase, disrupting ATP supply by mitochondria. Small decreases in ATP levels lead to apoptosis while large decreases rapidly cause necrosis (Murphy, 1999). NO mediated apoptosis is implicated in many neurodegenerative diseases, including Alzheimer's, Parkinson's and cell death in cerebral ischemia (Nicotera et al., 1995; Pai et al., 1998). Combining with certain cytokines, NO is a primary mediator of apoptotic cell death in osteoblasts (Damoulis and Hauschka, 1997).

NO at sufficient levels depresses myocardial contractility and is toxic to cardiac monocytes (Ing et al., 1999). By causing enterocyte apoptosis, NO produces “bare areas”
in the intestinal epithelium which are then susceptible to bacterial invasion and a subsequent systemic inflammatory response (Nadler et al., 1999). The dual role of nitric oxide both in endothelial cell apoptosis and survival has been observed. Endothelial cells undergo apoptosis via the mitochondria-dependent pathway that is regulated by NO production. NO-regulated endothelial cell injury thus may play a role in the disruption of vessel endothelium and contribute to the anti-endothelial cell antibody (AECA)-induced pathogenesis of vasculopathy (Lin et al., 2004).

Apoptosis is an important mechanism by which NO may contribute to the pathogenesis of SLE and can be implicated in SLE pathogenesis in at least three different ways:

1. As antigen, apoptotic material drives autoimmune responses.
2. As immune modulator, impaired apoptosis makes patients susceptible to developing autoimmunity.
3. As effector mechanism, apoptosis participates in target organ injury.

The relationship of apoptosis to SLE, however, cannot fit into a simple platitude such as "SLE is a condition of inadequate apoptosis," or "SLE is a condition of excess apoptosis." Rather, roles for both "too much" and "too little" apoptosis have been found. Moreover, "too much" and "too little" apoptosis can occur at the same time in the same SLE patient (Greidinger, 2001).

Conversely, NO demonstrates a protective effect against actinomycin induced liver apoptosis in mice (Akahori et al., 1999). Thus, depending upon the local tissue environment, NO may act as either a pro- or anti-apoptotic molecule (Stefanelli et al., 1999).

**Antioxidant Properties of Nitric Oxide**

Being a free radical, nitric oxide has both pro- and antioxidant properties (Hallman and Bry, 1996). NO can be protective against oxidative injury, depending on the specific conditions (Kanner et al., 1991). A nitric oxide radical can both stimulate lipid oxidation and mediate oxidant-protective reactions in membranes (Radi et al., 1991). At high rates of NO production, the pro-oxidant versus antioxidant outcome depends critically on the
relative concentrations of the individual reactive species (Rubbo et al., 1994). The pro-oxidant reactions of NO occur with superoxide, whereas the antioxidant effects of NO consequent to direct reactions with alkoyl and peroxyl radical intermediate during lipid peroxidation, terminating the propagation of lipid radical chain reactions (Rubbo et al., 1994).

Nitric oxide limits injury to target molecules or tissues during events associated with excess production of reactive oxygen species. These include inhibition of oxidative killing of murine lung fibroblasts and mesencephalic neurons (Wink et al., 1993b), attenuation of low-density lipoprotein oxidation (Graham et al., 1993; Hogg et al., 1993) and modulation (Kurose et al., 1994) and reduction of ischemia-reperfusion injury (Payne and Kubes, 1993).

Hydrogen peroxide mediates oxidation of different biological molecules that may result in tissue damage (Wink et al., 1993b). NO does not react directly with *OH, but is able to protect cells against *OH-mediated toxicity (Wink and Mitchell, 1998). NO induces ferritin, haem oxygenase, superoxide dismutase and endonuclease IV, which are protective proteins against oxidative stress, providing a cellular signal to up-regulate a variety of protective genes (Nunoshiba et al., 1993; Kim et al., 1995).

**Nitrate (NO$_3^-$) and Nitrite (NO$_2^-$)**

NO reacts with O$_2$ to form higher oxides of nitrogen, in a relatively slow reaction in places where there is high concentration of O$_2$. The essential fate of NO$^+$ is oxidation to (NO$_3^-$) and (NO$_2^-$), end products of NO metabolism that are rapidly distributed throughout the body and excreted in the urine. Overall, this oxidative metabolism of NO involves the formation of a number of intermediates in which the oxidation state of nitrogen ranges from +1 to +5 (N$_2$O nitrous oxide, N$_2$O$_3$ dinitrogen trioxide, NO$_2$ nitrogen dioxide, N$_2$O$_4$ dinitrogen tetroxide, N$_2$O$_5$ dinitrogen pentoxide) and several of these intermediates are presumed to be actively involved in the various biological actions of NO (van der Vliet et al., 1999).
Peroxynitrite (ONOO\textsuperscript{−})

Peroxynitrite is an important chemical species relative to the cytotoxic effect of NO. This compound, formed with NO and superoxide anion (Carreras et al., 1994) is a strong oxidant that damages cell membranes and proteins (Beckman \textit{et al}., 1990; Koppenol \textit{et al}., 1992; Matheis \textit{et al}., 1992; Yu \textit{et al}., 1994).

An immune cell that could release a great amount of NO and superoxide anion would be a major source of peroxynitrite (Ischropoulos \textit{et al}., 1992). It has been reported that endothelium also releases peroxynitrite through the stimulation of agonists (Kooy and Royall, 1994). The most serious bioactivity of the anion may be related to endothelial damage (Haddad \textit{et al}., 1993). Loss of the barrier of endothelium is often seen in the first stage of various oxidative damages in the biological system. The authentic ONOO\textsuperscript{−} or ONOO\textsuperscript{−} donor, SIN-1, clearly damages the endothelial cell (Beckman \textit{et al}., 1990). The nerve cell is also damaged by the anion (Lipton \textit{et al}., 1993; Oury \textit{et al}., 1993). The neuronal death is caused not by the NO molecule but by peroxynitrite (Lipton \textit{et al}., 1993). Some of the ONOO\textsuperscript{−} mediated pathological conditions include Parkinson's disease, arteriosclerosis, Alzheimer's disease, Huntington's disease, hypertension, multiple sclerosis, autoimmune myocarditis and others (Kuo \textit{et al}., 2000).

Protein Nitration

NO and its intermediates can not only cause nicks in the supercoiled plasmid DNA (Salgo \textit{et al}., 1995) but can also oxidize a variety of biomolecules including both proteins and non-protein thiols (Radi \textit{et al}., 1991), protein sulphides (Moreno and Pryor, 1992; Pryor \textit{et al}., 1994), lipids (Moncada \textit{et al}., 1991) and deoxyribose (Beckman \textit{et al}., 1990).

Peroxynitrite can modify proteins through oxidation of tryptophan and cysteine, formation of carbonyl moieties and cleavage of proteins (Ischiropoulos and Al-Mehdi, 1995). One persistent footprint left by peroxynitrite is nitration of phenolic rings, including tyrosine residues in proteins forming 'nitrotyrosine'. It can also be formed upon reaction of free or protein bound tyrosine with NO\textsubscript{2}+, NO\textsubscript{2}−, HONO and NO\textsubscript{2}Cl (Shigenaga \textit{et al}., 1997). Even cigarette smoke can nitrate tyrosine (Halliwell, 1997).
Nitrotyrosine has been identified as a stable end product and marker of inflammation and NO production. The mechanism of the formation of 3-nitrotyrosine and dityrosine are shown in Figure 4 and 5. Nitrotyrosine serves as a long term indicator of NO-mediated protein modification as the measure of NO production can be falsely elevated by exogenous dietary and medication sources of nitrite and nitrate and reduced by serum thiols (Oates et al., 1999). If significant NO production occurred two weeks ago, then returned to baseline, the level of nitrotyrosine in serum may still be elevated due to the long half life of some serum proteins (Gilkeson et al., 1998). Tyrosine nitration can inactivate enzymes and receptors that depend on tyrosine for their activity (Ye et al., 1996), e.g. E.coli glutamine synthase (Jiao et al., 2001), prostacyclin synthase (Zou et al., 1997) and cytochrome P450 2B1 (Roberts et al., 1999). Nitration also prevents phosphorylation of tyrosine residues important for signal transduction. Nitrotyrosine levels have been observed in injured tissues by both immunohistochemical techniques and qualitative analysis with HPLC or gas chromatography and mass spectrometry (Pfeiffer et al., 2001). Protein nitration occurs in a variety of cardiac and vascular disease states (Mihm et al., 2000). It may also be an important contributor to organ dysfunction disease (Ischiropoulos and Al-Mehdi, 1995; Zou et al., 1997).

Elevated nitrotyrosine levels have been detected in patients during renal failure, chronic smoking, sepsis and atherosclerotic plaques (Fukuyama et al., 1997; Petruzzelli et al., 1997; Leeuwenburgh et al., 1997). The levels of nitrotyrosine are also elevated in patients with rheumatoid arthritis and have also been observed in patients with celiac disease as well as SLE (Gilkeson et al., 1998; Oates et al., 1999). Nitrotyrosine has been detected from lung sections of patients and animals with acute lung injury (Haddad et al., 1994; Kooy et al., 1995), idiopathic pulmonary fibrosis (Saleh et al., 1997) and acute respiratory distress syndrome (ARDS) (Sittipunt et al., 2001). Peroxynitrite degrades surfactant by causing nitration of its tyrosine residues of surfactant proteins, formation of lipid peroxides and loss of surface activity (Haddad et al., 1993). Plasma nitrotyrosine was elevated in premature infants who developed chronic lung disease (CLD) (Banks et al., 1998). The presence of superoxide, transient metal, high concentrations of NO and oxygen and the absence of thiol groups, urate and ascorbate in the airways promote the destructive role of NO, as the generation of ONOO−
Fig. 4. Mechanism of reaction of ONOO\textsuperscript{−} with tyrosine. Peroxynitrite (ONOO\textsuperscript{−}) reacts with tyrosine in a first-order reaction via an electron transfer mechanism to form tyrosyl radicals and nitrogen dioxide (NO\textsubscript{2}\textsuperscript{−}) as products (Reaction 1). Subsequent radical combination reactions then yield 3-nitrotyrosine and 3,3\textquotesingle-dityrosine as products (Reactions 2 and 3) (van der Vliet et al., 1999).
Fig. 5. Hypothetical mechanism of 3-nitrotyrosine and dityrosine formation by peroxynitrite (Pfeiffer et al., 2000).
is accelerated or the defence mechanisms against ONOO⁻ toxicity are weakened (Eiserich et al., 1994; Hallman and Bry, 1996). In the past six years, nitration in at least eighty different diseases has been described (Reiter et al., 2000).

Nitric Oxide Scavengers

Overproduction of NO has been implicated in a number of diseases. There are two approaches to reducing NO levels: inhibition of NO synthase (NOS) or the binding and scavenging of NO in vivo. Aerobic organisms have potent antioxidant defenses whose role is to neutralize and minimize the potentially cytotoxic and genotoxic effects to reactive oxidants. Antioxidants are key line of defense capable of scavenging free radicals by preventing radical formation, intercepting radicals from further activity (Cotgreave et al., 1988), or participating in repair of damage caused by free radicals. Regulation of the antioxidant capacity includes the maintenance of adequate levels of antioxidant and the localization of antioxidant compounds and enzymes. Control over the activity of prooxidant enzymes, such as NADPH oxidase and NO synthases, is crucial. Synthetic antioxidants mimic biological strategies (Sies, 1993). Antioxidant defences may be primary or secondary. The defences that directly scavenge, H₂O₂ and 'OH are known as primary antioxidant defence. Secondary antioxidant defences consist of the repair mechanisms that act on biomolecules that have undergone oxidative damage. There may be enzymatic or non-enzymatic antioxidant defences. Enzymatic antioxidant defences include superoxide dismutase (SOD), catalase, gluththione peroxidase etc, whereas ascorbic acid, uric acid, glutathione are some non-enzymatic antioxidants distributed in biological systems. 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoine-1-oxyl 3-oxide (Carboxy-PTIO) is an efficient nitric oxide scavenger capable of trapping nitric oxide immediately after its synthesis. Therefore, carboxy-PTIO can be used as one possible treatment agent against various diseases that involve nitric oxide. Free nitric oxide can be inactivated by scavenger chemicals such as methylene blue, fuscidic acid, and carboxy-PTIO. The inhibitory effect of carboxy-PTIO on NO is found to be two-fold stronger than those of nitric oxide synthase inhibitors (Akaike et al., 1993; Wolfe and Dasta, 1995; Garcia-Pascual et al., 2000).
Autoimmunity

Autoimmunity is fundamentally a continuously evolving process. The autoimmune responses shift, drift and diversify with time not only to other epitopes in the original antigen but also to other related and sometimes unrelated antigens (Singh, 2004). The patients who have systemic autoimmune diseases become primed to recognize intracellular antigens. How the autoantibodies thus produced contribute to the pathogenesis of the disease, and how these autoantibodies access their target proteins. Normally, individuals do not form potentially destructive antibodies to their own cell components, but only to foreign antigens. This is because the body has developed a tolerance to the antigens (other than immunoglobulins) normally present within self. This state of the immune tolerance to self antigens is maintained by a complex network of T and B lymphocytes and their regulatory products. However, in some cases, the intracellular autoantigen targets of many systemic autoimmune diseases become altered during apoptosis in ways that may change how they are perceived by the immune system. High concentrations of self-antigens, or in the case of viral infection, complexes of foreign and self-antigens, are packaged during generation of apoptotic cells. The packages also may contain altered fragments of self-antigens that have not been encountered previously by the immune system. Under normal circumstances, macrophages and dendritic cells clear apoptotic cells rapidly. The normal consequence of that clearance is that the apoptosis-altered self-antigens are either ignored by the immune system or tolerance to those antigens is maintained. Defects in this process that cause a delay in clearance could change the appearance of apoptotic cells and cause them to be recognized as "foreign invaders," thereby stimulating an inflammatory response that, in turn, activates an immune response to self-antigens (Navratil et al., 2004). One possible explanation for the occurrence of autoimmune response is that the T-cell repertoires of patients include auto reactive clones that have escaped thymic selection (Herold, 2004). Autoantibodies can also be produced against soluble self-antigens, which can form immune complexes. This might lead to activation of the complement cascade, with the formation of the anaphylactic and chemotactic fragments, C3a and C5a. Histamine is then released and phagocytic activity is increased, causing an
inflammatory response that is destructive to tissues at the site of immune-complex formation. This is the usual series of events during acute episodes in SLE, when auto-antibodies to DNA generate anti-DNA: DNA complexes with subsequent inflammation. There are many more ways in which an autoimmune response can cause tissue damage. General mechanisms of action can be classified into the following groups:

1. Damage by complement-fixing antibodies raised against auto-antigens.
2. Compromise of cellular function when autoantibodies bind to the cell surface receptors, which mediate, degrade or block expression of differentiated function.
3. Tissue damage when autoantibodies and soluble self-antigens form immune complexes and initiate a destructive inflammatory response.
4. Damage to cells through specific T cell responses activated to destroy self-cellular antigens.

Autoimmune diseases are both multigenic and multifactorial in etiology. The triggers for autoimmune diseases are diverse and include genetic, immunologic, hormonal and environmental, acting singly or in combination, in time and space (Brickman and Shoenfeld, 2001). At present many individual mechanisms have been identified, but how they interact with the immune network has not yet been elucidated.

**Autoimmunity and Genetic Factors**

Autoimmune diseases show a highly familial predisposition. Clinicians treating patients with autoimmune disorders have been long struck by the finding that such patients frequently have relatives with the same or with other autoimmune disorders (Shoenfeld and Isenberg, 1989). Majority of genes which have been associated with autoimmune diseases map within the major histocompatibility complex (MHC) of man, or HLA region, particularly the HLA-DR and DQ sublocus. The HLA genes function as secondary genes to allow expression of specific autoantibody or respective disease state. The HLA molecules that are present on the surface of all nucleated cells and platelets are encoded for within the MHC on the short arm of chromosome 6 in humans. The initiation of an autoimmune response requires that the self reactive T cells interact with self antigen and HLA II antigen complex with sufficient avidity for the development of autoantibody
in subsequent diseases (Bias et al., 1986; Goldstein and Arnett, 1987; Braun and Zachary, 1988; Arnett and Moulds, 1991; Deodhar, 1992; Colbert, 2000).

In both animals and humans, autoimmune diseases have also been linked to non-MHC genetic factors. Fcγ receptor phenotypes (Oh et al., 1999), DNAse I (Walport, 2000) and serum amyloid protein Sap genes (Walport, 2000) as well as several apoptosis associated genes (Oh et al., 1999) are few of the non-MHC factors suspected of predisposing to autoimmune diseases.

Autoimmunity and Immunologic Factors

Autoantibodies may be humoral or cell mediated, or a combination of both. B cells contribute more than antibody production to the development of autoimmune disease, though possessing receptors for self antigens, e.g. thyroglobulins, DNA and IgG, they do not normally produce significant quantities of antibodies unless they receive T cell help. B cells amplify an immune response through their function as antigen-presenting cells. Epitope spreading, or the recognition of an increasing number of antigenic determinants on a molecule or molecular complex, is an important mechanism in autoimmunity (Davidson and Diamond, 2001). This process involves B cells and T cells (Nossal, 1994; Ashton-Rickardt and Tonegawa, 1994; Von Boehmer, 1994; Weinstein et al., 2004). In a wide variety of autoimmune diseases, the regulatory failure results in a significant decrease in T-suppressor cell numbers and activity, thereby unbalancing the T-helper/T-suppressor cell ratio. The increased T-helper/T-suppressor cell ratio has been noted in a wide variety of autoimmune diseases, such as SLE, Sjogren’s syndrome, PSS or scleroderma, rheumatoid arthritis, pernicious anemia, multiple sclerosis, immune complex mediated renal diseases, immunologic skin diseases and many others. Immunologic cross reactivity and molecular mimicry has been an important phenomenon in autoimmune diseases (Prinz, 2004). It has been suggested that the degree of sequence conservation between host and given infectious agent, heat shock proteins, because of molecular mimicry, may provide the link between infection and subsequent autoimmunity.
Autoimmunity and Hormonal Factors

Hormones as well seem to influence the expression of certain autoimmune diseases. It is known that hormones of the hypothalamus, thyroid and adrenal glands affect the homeostasis of the lymphoid system and responses to antigens, by as yet uncharacterized mechanisms. SLE and RA preferentially afflict women, where as more men develop myasthenia gravis. The predisposing factors in these instances appear to be the sex hormones. It is known that testosterone is immuno-enhancing. Other hormones including progesterone and prolactin appear to have immunoregulatory properties. How these hormones contribute to the disease state has not been elucidated (VanVollenhoven and McGuire, 1994; Walker et al., 1998; Ahmed and Talal, 1990; Lahita, 1999).

Autoimmunity and Environmental Factors

Environmental factors have been implicated in autoimmune diseases including infectious agents, medications, chemicals, toxins, and ultraviolet light (Aharon-Maor and Shoenfeld, 1998; Saraux et al., 1999). Ultraviolet light is known to trigger SLE (McGrath, 1999). D-penicillamine, a medication formerly used to treat several immune diseases including RA, scleroderma and primary biliary cirrhosis has been implicated in the development of autoimmune diseases such as SLE and glomerulonephritis (Brik et al., 1995). Malignancies are common in most autoimmune diseases than in general population, i.e. lymphomas are 30-40 times more common in primary Sjogren’s syndrome (Ramos-Casals et al., 2000) and, conversely, autoimmune manifestations are a relatively common paraneoplastic manifestation, i.e. vasculitis, sensory neuronopathy or autoimmune encephalomyelitis (Graus et al., 1986).

Role of Nitric Oxide in Autoimmunity

There has been conclusive evidence that NO can participate as a mediator of tissue damage in autoimmune diseases (Table 4). Nitric oxide does not participate in the inducing phase but in the effector phase of the autoimmune response (Cross et al., 1994). Macrophages and PMN cells appear to be present in autoimmune inflammatory lesions
### TABLE 4
Implication of Nitric Oxide in some Autoimmune Diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Species</th>
<th>Treatment with NOS Inhibitor</th>
<th>Nitric Oxide or Nitrite Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental allergic encephalomyelitis (EAE)</td>
<td>Mouse, Rat</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Experimental neuritis (Adoptive)</td>
<td>Rat</td>
<td>Yes</td>
<td>-----</td>
</tr>
<tr>
<td>Diabetes spontaneous</td>
<td>Mouse, Rat</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Diabetes, induced by streptozotocin</td>
<td>Mouse</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Lupus like syndrome</td>
<td>Mouse (MRL-lpr/lpr)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Arthritis</td>
<td>Mouse, Rat</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Man</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>Man</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
and can even have a major role in cell damage (Vladutiu, 1995). These cells are well known to produce NO once NOS is induced in these cells by cytokines. There is a release of cytokines in autoimmune inflammation and their role in autoimmune diseases is becoming more apparent. Finally, in some autoimmune diseases, immune complexes may play a pathogenic role and it has been reported that immune complex deposition can lead to NO release (Mulligan et al., 1991). Devlin et al. (1994) have reported nitric oxide generation as a predictive parameter of acute allograft rejection. Genetically determined over expression of NO plays a role in etiology of autoimmunity and may also serve as a barrier to transplantation (Mills et al., 1994). It appears that cytokines are very important for NO production in autoimmune diseases, and cytokines are present in increased amounts in autoimmune lesions where they are produced by inflammatory cells (Kroemer and Martinez, 1991). A potential source of NO is Th1 lymphocytes, as has been shown by Taylor-Robinson et al. (1993) in experimental malaria infection. In addition to lymphocytes (Kirk et al., 1990), human neutrophils (Moncada, 1992) and chondrocytes (Blanco et al., 1995) can generate NO after induction of NOS. Another possible source of NO is endothelial cells, and a common early feature of the autoimmune lesion is endothelial cell swelling, leading to the formation of high endothelium venules (Miossec, 1993). Infiltrating macrophages, resident endothelial cells, astrocytes (Hewett et al., 1993) or microglial cells (Zielasek et al., 1992) in the central nervous system of animals with EAE may secrete NO (Lin et al., 1993).

Autoimmune diseases can be classified into two broad but overlapping groups: organ specific and non-organ specific (or systemic) autoimmune diseases. Grave’s disease, Hashimoto’s thyroiditis, pernicious anemia, Addison’s disease, insulin dependent diabetes mellitus, rheumatoid arthritis, autoimmune hemolytic anemia, autoimmune gastritis are a few examples of organ specific diseases. Autoantibody or cell mediated reactions against a specific target antigen, located in a specialized cell, tissue or organ occurs. In systemic autoimmune diseases, by contrast, tissue injury and inflammation occur in multiple sites in organs, e.g., Sjogren’s syndrome, systemic sclerosis, mixed connective tissue disease, systemic lupus erythematosus and antiphospholipid syndrome.
Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a complicated autoimmune disease at both the phenotype and genotype levels in humans and in experimental mouse models. It is one of the most serious rheumatic diseases. According to a 2002 government study, the annual number of deaths had risen from 879 to 1,406 since 1979. About a third of these deaths occurred in people aged 15 to 44 years, mostly women. The disease, however, is unpredictable and varies greatly from one individual to the next. Severity also appears to differ among ethnic groups and countries. In Europe and North American SLE patients, for example, overall five-years survival rates are between 93% and 95%, while in Asia or Africa they are considerably lower (60% to 70%). Other research also indicates that in the US African-American and Hispanic patients suffer greater organ damage than Caucasian patients. Genetic factors appear to have some influence on specific effects of SLE on organ damage among ethnic groups. The poorer outlook among minority groups and in underdeveloped nations appears to be primarily due to less access to good health care.

There is a genetic predisposition for SLE supported by family studies, which show sibling risk of 5-10% and identical sibling risk of >25%, whereas the population risk is probably closer to 0.1%. It is a prototypic autoimmune disease that afflicts predominantly women during their child bearing age (Nambiar, 2002). Identification of genetic factors that contribute to susceptibility to SLE in human and mouse is difficult due to several reasons including disease heterogeneity and complex interactions of hormonal, genetic and environmental factors (Tsao, 2003; Scofield, 2004; Santiago-Raber et al., 2004). SLE is characterized by the production of multiple autoantibodies, typically antinuclear and anti-DNA antibodies (Tan, 1989; Davidson et al., 1990; Steinberg 1992; Harada et al., 1994; Petri, 1996). In some patients antibodies are also produced against platelets, lymphocytes, cellular antigens such as polysaccharides (Kashiwara et al., 1993), phospholipids (Tiikkainen et al., 1991), cell membrane structures (Jacob et al., 1986a,b and c) and nucleoproteins (native nucleosome, its DNA component and/or its histone component), nuclear ribonucleoproteins like Sm and Mo, cytoplasmic ribonucleoproteins like Ro/SSA and La/SSB (Stollar, 1975, 1980, 1981; Gabler et al., 2003). The clinical manifestations include fever, an erythematosus
‘butterfly rash’ across the face, lesions of discoid lupus or a vasculitic rash, polyarthralgia and arthritis, polyserositis (especially pleurisy and pericarditis), anemia, thrombocytopenia, renal, neurologic and cardiac abnormalities. One of the impediments in understanding human systemic lupus has been its marked heterogeneity (Steinberg et al., 1991; Steinberg, 1992). The development of a fatal immune complex (IC)-mediated glomerulonephritis (GN) associated with immunological abnormalities such as autoantibody production makes several animal models close enough to human SLE. Systemic Lupus Erythematosus is characterized by the formation of a variety of autoantibodies and subsequent development of severe glomerulonephritis. SLE is characterized by the development of T cell and antibody responses against a variety of self antigens and inflammation in multiple organs (Malaviya et al., 1988; Mills et al, 1994; Hahn, 1998). The disease usually begins with the involvement of a few organs and gradually evolves into a multisystem disorder involving the skin, joints, brain, lungs and kidneys. The disease is characterized by immune dysregulation, leading to high level autoantibody production; immune complex deposition and vasculitis (Parke and Rothfield, 1985). The autoantibody pattern also diversifies with time (Scofield et al., 1996). However, not all patients with SLE develop all autoantibodies or involvement of all organs. Evidence suggests that the initial autoimmune response in SLE is restricted to a few epitopes (Singh, 2004). In SLE, the phenomenon of epitope spreading offers an explanation for the development of autoantibody specificities to antigens that are part of the same molecular complex, such as the close association of anti-Ro and anti-La antibodies (Weistein et al., 2004).

The initial immunizing antigen(s) that drive the development of SLE are unknown, but characteristics of the immune response in SLE suggest that it is an antigen-driven condition. A number of phenomena have been observed after inoculation with known antigens that distinguish antigen-driven immune responses from other patterns of immune activation. These include oligoclonal expansion of antigen-specific cells, up-regulation of parallel B and T cell responses against the same antigen, epitope spreading to progressively more diversified structures on the antigen and selection for immune cells with higher affinity for the antigen (Greiding, 2001). In SLE, all of these phenomena have been identified. Immunity in SLE is directed against self antigens not targeted in
normal individuals, suggesting a failure of self-tolerance (Feuerstein et al., 1999). Apoptosis plays a crucial role in the regulation of the immune system. Disruption in apoptotic pathways can prevent the deletion of autoreactive cells (Kurts et al., 1998), and impair the resolution of ongoing inflammatory responses (Lee et al., 1988). Some such disruptions have been associated with increased risk for the development of SLE. Impaired apoptosis can be caused by deficiencies in endogenous pro-apoptotic mediators, over-expression of endogenous anti-apoptotic mediators, or by acquired factors. In autoimmune diseases including systemic lupus erythematosus, the immune system attacks various autoantigens and causes damage in target organs. Recently, it has been found that dead cells serve as a repertoire for autoantigens which can stimulate an autoimmune response in sensitive persons. The mechanisms, which lead to induction and progress of apoptosis, include extra-cellular stimuli, intra-cellular signals, and cleavage of proteins. During apoptosis, several events occur including migration of intra-cellular components to the cell membrane, removal of apoptotic cells by specific proteins and complement systems, and phagocytosis of apoptotic cells by macrophages (Marai et al., 2003).

Environmental triggers such as viruses have been provisionally linked to the development of SLE, e.g. Epstein-Barr virus and adenovirus (James et al., 1997), human T cell lymphotropic virus (Brand et al., 1999), hormonal and chemical exposure (Cooper et al., 1998), support a gene-environment interaction, and one cohort-comparison study supports a gene-gene interaction (Mehrian et al., 1998). SLE is highly variable among individual patients and, to some extent, between ethnic groups since certain disease manifestations seem to cluster within racial populations whereas others do not (Petri, 1998).

Deficiencies in the classical pathway of the complement system have been implicated in the etiology and pathogenesis of systemic lupus erythematosus for several decades (Liu et al., 2004). Complement has both beneficial and deleterious roles in the pathogenesis of systemic lupus erythematosus. On the one hand, patients with SLE present with decreased complement levels (C1, C2 and C4) and with complement deposition in inflamed tissues, suggestive of a harmful role of complement in the effector phase of disease. On the other hand, homozygous deficiency of any of the classical pathway proteins is strongly associated with the development of SLE. There are two main
hypotheses to explain these observations. The first invokes an important role for complement in the physiological waste-disposal mechanisms of dying cells and immune complexes. The second hypothesis is based around the role of complement in determining the activation thresholds of B and T lymphocytes, with the proposal that complement deficiency causes incomplete maintenance of peripheral tolerance (Manderson et al., 2004). Excessive complement activation as a result of a regulator component deficiency leads to tissue injury that mimics that seen in autoimmune disease. Complement activation occurs during tissue injury and contributes in a major way to the expression of pathology. It appears that natural antibodies represent an early culprit in tissue injury following ischemia reperfusion injury. Natural antibodies and probably autoantibodies present in sera of patients with systemic autoimmune disease bind to tissues already exposed to a damaging insult, activate complement and produce pathology (Tsokos and Fleming, 2004). Polymorphisms in low affinity IgG (Fcγ) receptors, which are important for the clearance of immune complexes, are also implicated in the pathogenesis of lupus (Salmon et al., 1996; Wu et al., 1997).

Systemic lupus erythematosus is also characterized by B cell hyperactivity in association with autoantibodies, most prominently those directed to components of the cell nucleus. The source of the antigens that drive B cell responses in SLE is unknown, although recent studies suggest mechanisms by which the self-antigens become immunogenic and stimulate responses. In addition, autoantibody crossreactivity may promote induction of responses to disparate antigens, foreign and self, and enable a single autoantibody to cause disease by cross-reactive binding. In addition to reflecting increased exposure to self-antigen, autoantibody responses in SLE may result from abnormalities in B cell signaling and regulation by cytokines (Criscione and Pisetsky, 2003).

B-cell activation generally requires T-cell help. T cells have been cloned from lupus prone mice and these were found to stimulate the production of anti-DNA antibodies and renal lesions when injected. Obligatory and enhanced T cell help for B cells is shown in SLE lymphocytes by prolonged expression and co-stimulatory interaction of the helper T cell surface ligand. CD40 (CD40L) with B cell receptor CD40 (Desai et al., 1996; Koshy
The interaction of CD40L on activated T cells with CD40 on B cells, induces B cell proliferation and formation of germinal centers. Within germinal centers, further cell to cell interactions leads to B cell maturation through immunoglobulin isotype switching, somatic mutation, clonal expansion of high affinity B cells and terminal differentiation to plasma cells (Reiser and Stadecker 1996; Lindhout et al., 1997; Tarlinton et al., 1998). Blockade of CD40L has been shown to delay the onset of disease in SLE-prone mice and to stabilize or reverse existing renal disease (Mohan et al., 1995; Kalled et al., 1998). Recent studies in SLE-prone mice (NZB x NZW) F₁ show that an anti-CD40L monoclonal antibody administered to pre nephritic mice inhibits both T cell activation and T cell dependent B cell activation (Huang et al., 2002).

T cell abnormalities, B cell hyperactivity and abnormal cytokine production have been implicated to be of pathogenic importance in SLE. SLE patients have an increased production as well as increased serum level of the type 2 cytokines IL-10 and IL-6. Serum IL-10 levels correlates with the titre of anti-dsDNA antibodies in SLE patients (Grondal et al., 2000). The role of these cytokines in the pathogenesis of SLE is unknown theoretically, IL-10 could stimulate peripheral blood mononuclear cells of SLE patients to produce autoantibodies, and thus be important in the excess autoantibody production that is characteristic of SLE (Llorente et al., 1995). High IL-10 production is reported in healthy relatives of SLE patients in multi case families, indicating that this tendency may be a genetic factor in pathogenesis (Llorente et al., 1997).

Based on intensive research during the last decades, three main mechanisms might contribute to the development of SLE (Lorenz et al., 2001).

1. Increased amounts and abnormal presentation of potential autoantigens including nuclear antigens.
2. T cell dependent stimulation of B cells for the production of antinuclear antibodies.
3. Anti dsDNA as well as immune complex mediated organ damages.

**Role of Oxygen Free Radicals in SLE**

Oxygen free radicals are important in both natural and acquired immunity. Neutrophil and macrophage phagocytosis stimulates various cellular processes including
the "respiratory burst" whereby increased cellular oxygen uptake results in the production of the potent oxidant bactericidal agents (Knight, 2000), thereby killing bacteria and regulating the process of acute inflammation (Halliwell, 1982). Inflammatory response is thus advantageous for the organism. However, abnormal over activation of phagocytes with consequent exacerbation of reactive oxygen metabolites production may damage surrounding tissues and lead to diseases such as SLE (Proctor and Reynolds, 1984; Southorn and Powis, 1988; Halliwell and Gutteridge, 1989). In vivo, ROS are generated by oxidant enzymes, phagocytic cells, ionizing radiation, etc. Superoxide anion is believed to be the first radical formed, mainly by the electron transport chain when O₂ picks up the single electron (Ahsan et al., 2003). ROS are implicated in the inflammatory, autoimmune, connective tissue disease, systemic lupus erythematosus, particularly in respect of processes leading to the formation of pathogenic anti-DNA antibodies. Damage due to inflammatory processes is seen more often in systemic diseases than organ specific diseases. Accumulating evidence suggests that ROS mediate apoptotic cell death as intracellular ROS levels increase and cells undergo apoptosis (Gardner et al., 1997; Lelli et al., 1998; Lieberthal et al., 1998). Recent studies show that mitochondrial hyperpolarization, increased ROS production and cytoplasmic alkalinization plays crucial roles in altered IL-10 responsiveness in SLE (Gergely, 2002). ROS generation through normal cellular metabolism and by exogenous stimulus is a constant problem for which cells have developed multiple defense mechanisms to survival. An imbalance between free radical generation and sequestration leads to oxidative stress (Ahsan et al., 2003). There is growing evidence for a role of oxidative stress in the etiology and pathogenesis of SLE (Evans et al., 2000). DNA is a major target for oxidants resulting in increased autoantigen production, enhancement of antigenicity and altered cell functions (Blount et al., 1990; Ara and Ali, 1992; Cooke et al., 1997).

It has been proposed that in chronic inflammatory diseases such as RA and SLE, DNA-anti-DNA antibody complex(es) deposit in tissues and induce inflammation (Naparstek and Madaio, 1997). The phagocytic cells may then release ROS at the site of injury (Allan et al., 1988). These oxygen species being highly reactive may penetrate cellular membranes and react with nuclear DNA (Allan et al., 1987; Stollar, 1981). It has been reported that anti-DNA antibodies found typically in SLE have greater capacity to
bind to ROS-modified DNA (Blount et al., 1989). Recent studies have demonstrated that after modification with ROS, DNA becomes highly immunogenic and the induced antibodies exhibit variable binding to native DNA (Ahmad et al., 1997; Ashok et al., 1997; Ashok and Ali, 1999). It has been postulated that ROS modified DNA is more discriminating antigen for the diagnosis of SLE than native DNA (Blount et al., 1990; 1991, Ara et al., 1992; Ara and Ali, 1993). Defective DNA damage processing has been reported in SLE. Lymphocytes isolated from patients suffering from SLE contain increasing levels of 8-oxodG in DNA (Bashir et al., 1993), a marker of oxidative damage. An increase in serum 8-oxodG were detected in SLE patients implying a defect in the processing of 8-oxodG in SLE (Evans et al., 2000).

Protein structure and thus functions are also modified by ROS. Metal ion catalyzed protein oxidation results in addition of carbonyl groups, cross-linking and fragmentation. Aldehydes of lipid peroxidation can react with sulphhydril (cysteine) or basic amino acids (histidine, lysine) affecting their biological characteristics. Similarly, modification of individual nucleotide bases, single strand breaks and cross-linking are the typical affects of ROS on nucleic acids (Aruoma et al., 1991).

Recent studies from our laboratory have been carried out to synthesize and characterize the photoconjugates between positively charged amino acids (lysine and arginine) and the polydeoxyribonucleotide C [poly(dC)]. A strong recognition of photoadducts was observed with anti-DNA autoantibodies found in the sera of SLE patients. These photoadducts were found to be effective inhibitors and their relative affinity was substantially higher than native poly (dC). These results have pointed to the likelihood of modifications to polynucleotides in DNA for its better recognition by SLE autoantibodies as a causative agent for the induction of circulating anti-DNA or anti-polynucleotide antibodies. Therefore, it appears that lysine and arginine have a pivotal role in the generation of these antibodies (Dixit et al., 2003).

Native calf thymus DNA and poly(dA-dT).poly(dA-dT) have also been photoadducted with 8-methoxypsoralen inducing formation of interstrand photo-crosslinks. It has been found that crosslinked species of DNA-8-M0P and poly(dA-dT)-8-MOP photoadducts recognize previously defined monoclonal anti-Z-DNA antibody. These studies pointed out that conformational changes in DNA arising from the photo-
addition might have provided neoantigens for the induction of autoantibodies to DNA which in turn has resulted in autoimmune disease (Arif and Ali, 1996).

It has now been established clearly that not only oxygen but also nitrogen free radicals play an important role in the pathogenesis of several human diseases. Reactive nitrogen species is produced by the reaction of nitric oxide with O$_2^{$*}$ or peroxide. Nitric oxide radical participates in some pathological conditions such as arthritis, autoimmune diseases, vasculitis, asthma, hypertension, etc. It is also an unstable molecule, like ROS but less reactive, and can react with proteins, O$_2$ and O$_2^{$*}$ (Ahsan et al., 2003).

**Role of Nitric Oxide in SLE**

There is increasing evidence that nitric oxide (NO) may be important in the pathogenesis of systemic lupus erythematosus (SLE). Studies using N$^8$-monomethyl-L-arginine in MRL-lpr/lpr mice indicate that nitric oxide is important in the pathogenesis of glomerulonephritis, arthritis and vasculitis (Weinberg et al., 1994; Oates et al., 1997). NO is an important mediator of the inflammatory response. The work of Gilkeson et al. (1997), studying inflammation in MRL-lpr/lpr mice with genetically disrupted NOS2, highlights the heterogeneity and complexity of the role of NOS2 and nitric oxide. MRL-lpr/lpr mice over express NOS2 and over produce nitric oxide, parallel with the development of autoimmune syndrome with a variety of inflammatory manifestations. Previous studies have shown that nitric oxide production with nonselective NOS inhibitor N$^O$-monomethyl-arginine reduced glomerulonephritis, arthritis and vasculitis. To further define the role of nitric oxide and NOS2 in disease, mice with targeted disruption of NOS2 were produced by homologous recombination. Measures of nitric oxide production were markedly decreased in the MRL-lpr/lpr (-/-) mice (homozygous for disrupted NOS2), compared with MRL-lpr/lpr (+/+)(wild type), with intermediate production by the MRL-lpr/lpr (+/-) mice (heterozygous for disrupted NOS2). One possible explanation for the differences observed in NO production between SLE patients and controls is variation in the 5' promoter region of the NOS2 gene, which controls NOS2 transcription (Lopez-Nevot et al., 2003).
Retrospective studies have suggested a role of NO in the pathogenesis of SLE by demonstrating elevated levels of NO in these patients. Murine models of SLE demonstrate abnormally high levels of NO compared with normal mice, while systemic blockade of NO production reduces disease severity. Native DNA per se is a weak immunogen, while its modified forms have been shown to be immunogenic. Recent studies from our laboratory have demonstrated that after modification with ROS and NO, DNA became immunogenic and the induced antibodies exhibited variable binding to native DNA. Nitric oxide modification exposes base residues in the DNA backbone and the minor regions of single-stranded DNA (ssDNA), rendering it highly immunogenic (Dixit and Ali, 2004). Two potential sources of excessive NO are activated endothelial cells and keratinocytes via up regulated NOS2 (Belmont et al., 1997). Serum nitrite/nitrate level, which is an index of nitric oxide production, was found to correlate with the disease activity and with the levels of antibodies to dsDNA. Subjects with active lupus nephritis had higher levels of serum nitrotyrosine than those without renal disease, suggesting that overproduction of NO may play a pathogenic role in SLE and lupus nephritis (Gilkeson et al., 1998; Oates et al., 1999). The significance of oxidative stress and NO and antioxidants in SLE has also been reported (Mohan and Das, 1997). Eicosapentaenoic acid and docosahexaenoic acid (essential fatty acids) can modulate oxidative stress and nitric oxide synthesis and may have a regulatory role in the synthesis of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase. They have also suggested that measurement of lipid peroxides, nitric oxide and anti-oxidants can be used as markers to predict prognosis in patients with SLE.

Role of 3-Nitrotyrosine in SLE

A correlation between serum nitrate/nitrite (N/N) levels and serum nitrotyrosine levels with lupus disease activity has been observed. Serum N/N levels and serum nitrotyrosine levels, although both measures of NO production, may not necessarily correlate in individual patients at a given time. Serum N/N are direct metabolites of NO and are rapidly cleared from the serum. They thus reflect systemic NO production at that
time, similar in a way to measures of serum glucose in diabetes. Nitration of tyrosines, however, is irreversible and can be detected as long as the nitrated protein is in circulation. Serum nitrotyrosine levels reflect NO production over time, perhaps most analogous to serum glycosylated hemoglobin in diabetes (Gilkeson et al., 1998).

Peroxynitrite levels (as measured by 3-nitrotyrosine) correlate more significantly among African-Americans with disease activity than the frequently used measure of serum N/N. Lupus patients with no disease activity have similar sera 3-nitrotyrosine levels to control, suggesting that abnormal NO production occurs in disease flares only. The differential expression of N/N and 3-nitrotyrosine in Caucasian and African-American lupus subjects suggests that more oxidative stress (i.e., superoxide production) accompanies NO production among African-Americans with lupus. The increased peroxynitrite production in this group of patients may explain the more aggressive clinical expression of disease in African-American (Reveilli et al., 1990; Austin et al., 1994, 1995; Bakire et al., 1994; Alarcon et al., 1998; Petri, 1998; Reveilli et al., 1998).

Nitrated renal proteins have been demonstrated in murine lupus nephritis, which corresponds with increased nitrated serum proteins and abnormal NOS2 staining in the kidney. As 3-nitrotyrosine correlates with overall SLEDAI scores even in the absence of renal disease, the kidney cannot be the only source of NO production in SLE. Elevated N/N levels in the cerebrospinal fluid of patients with cerebral lupus and abnormal NOS2 expression in the keratinocytes and vascular endothelium of lupus patients in general have been seen. Wang et al. (1991) reported increased apoptotic activity among renal cells adjacent to those expressing NOS2 protein. Any or all of these mechanisms may contribute to NO-mediated renal damage in lupus. Free 3-nitrotyrosine and serum N/N are cleared renally, but serum protein 3-nitrotyrosine should be retained in those subjects without proteinuria. Levels of serum 3-nitrotyrosine could be falsely reduced in patients with severe renal protein loss. However, subjects with proteinuria have been found to have elevated serum 3-nitrotyrosine despite this increased protein loss. Thus, serum 3-nitrotyrosine is best defined as a measure of protein modification by nitric oxide (Oates et al., 1999).
Objectives of the Present Study

Nitration of tyrosine represents *in vivo* a mechanism, which can severely compromise the cell function. The detection of 3-nitrotyrosine in pathological tissues was suggestive of the occurrence of nitrating pathways and considered a possible diagnostic marker for reactive nitrogen species production *in vivo*. Protein nitration occurs in a variety of cardiac and vascular disease states. We tested the role of tyrosine nitration in the etiopathogenesis of systemic lupus erythematosus. The circulating human autoantibodies in this autoimmune disorder are polyspecific in relation to antigen binding characteristics. These antibodies not only recognize nucleic acid antigens but also react with a number of cellular proteins. Nitrogen free radicals play a significant role in health and disease.

In the present study, commercially available poly L-tyrosine was exposed to nitric oxide generated by sodium nitrite in acidic medium. The modified product was then characterized by UV and fluorescence spectroscopy, DNA adduct formation, thermal denaturation studies, gel filtration, electrophoresis and circular dichroism spectroscopy.

Polyclonal antibodies against native and nitric oxide modified poly L-tyrosine were generated in experimental animals. Both the native and modified samples induced high titre antibodies. However nitric oxide modified poly L-tyrosine was found to be more immunogenic in comparison to native poly L-tyrosine as assessed by direct binding ELISA. The specificity of induced antibodies was evaluated by competition ELISA, gel retardation assay and dot blot assay.

In order to assess the possible role of nitric oxide modified epitopes in the etiology of autoimmune diseases, sera from various SLE patients were studied for their recognition of native and nitric oxide modified poly L-tyrosine. In the sera of SLE patients, 3-nitrotyrosine has also been detected by western blot analysis. Separation of 3-nitrotyrosine was achieved by HPLC and the concentration was calculated in SLE patients and the serum from the experimental animals.