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DEDICATION

I dedicate this modest research work to:

My dear father "**Boudjemaa**," who I love very much and who have been doing everything possible for me, for all his efforts, encouragment and patience during all these years.

My dear mother "**Nassima,"** who I love very much and who has always hoped for my success and gave me enough affection. I pray for God to protect her and keep her safe from harm. I wish her a long life full of health and prosperity.

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To all my friends, I wish a life full of joy, happiness and health.

My partner**" Maissa"** and all her family.

Naila

DEDICATION

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ABBREVIATIONS

ACE: Angiotensin-converting enzyme

AD: Alzheimer's disease

ADMA: Asymmetric dimethylarginine

BH4: Tetrahydrobiopterine

CAM: Calmodulin

CAT: Cationic transporter arginine

CNS: The central nervous System

CRP: Protien C reactive

EDRF: Endothelium-derived relaxing factor

ENOS: Endothelial nitric oxide synthase

GCs: Guanylyl Cyclase

GMPc: cyclic guanosine-3′, 5′-monophosphate

GTP: Guanosine triphosphate

HDL: High-density lipoprotein

INOS: Inducible nitric oxide synthase

LDL: Low-density lipoprotein

L-NNA: NG-nitro-l-arginine

L-NAME: Methyl ester of NG-nitro-l-arginine

L-NMA: Nω-methyl-l-arginine

L-NMMA: N^G-monomethyl-L-arginine

L-NOHA: N-hydroxyl-l-argenine

LRRK2: leucine-rich repeat kinase 2

LTP: long-term potentiation

NADPH: Diaphorase activity

NF-κB: Nuclear factor-kappa B

NMDA: Methyl-D-aspartate

NNOS: neuronal nitric oxide synthase

NO: Nitric oxide

NOHA: N-omega-hydroxy-L-arginine

NOS: NO synthase

P: Parkinson's disease

PINK1: PTEN-induced putative kinase 1

PKG: dependent protein kinase

POLG1: mitochondrial polymerase gamma 1

RBC: Red Blood Celle

ROS: Reactive oxygen species

SMC: Smooth muscle cells

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Introduction

INTRODUCTION

Nitric oxide (NO) is a free radical that acts as a moiety of the endothelium-derived relaxing factor (EDRF) **(Ignarro *et al.*, 1987)**. The production of NO occurs in almost all mammals cells and tissues, including fat cells, brain, endothelium cells, neurons, neutrophils, hepatocytes, macrophages and skeletal muscles **(Wu and Meininger, 2002)**. Several functions of NO have been described. It acts as a novel transcellular, messenger molecule in many key physiological and pathological processes **(Moncada *et al.*, 1991)**.

NO plays a central role in the cardiovascular system as the endothelium derived relaxing factor **(Ignarro *et al.*, 1987).** Also, plays an important role in maintaining basal vascular tone through its relaxing effect on smooth muscle cells. In the healthy heart, the effect of NO on cardiac contractility depends on its concentration. Thus, at baseline, low concentrations of NO have a positive inotropic effect while at higher amounts it appears to be negative inotropic (**Massion *et al.*, 2003).** On the other hand, NO can inhibit creatine kinase, reduce the activity of the respiratory chain, and thus influence myositis metabolism **(Casey *et al.*, 2005).** NO has been shown to prevent neointimal hyperplasia by inhibiting the proliferation of vascular smooth muscle cells (**Marks *et al.*, 1995).** Additionally, NO may inhibit platelet aggregation and adhesion as well as monocyte chemotaxis and leukocyte adhesion to vessel walls (**Radomski *et al.*, 1992).**

In the central nervous system, NO is a crucial component of the signal transduction pathways used for memory formation, sensory processing, and the regulation of cerebral blood flow **(Xu and Liu., 1998).** NO plays various roles as a neurotransmitter, including the regulation of hormonal secretion and several cognitive functions. The maintenance of synaptic plasticity as well as the control of appetite, body temperature and cycle **(Calabrese *et al.*, 2007**). At the peripheral level, NO controls the relaxation of non-adrenergic non-cholinergic smooth muscle fibers and allows the stomach to adjust to larger volumes of food, regulate intestinal peristalsis and muscle tone of the intestinal sphincters **(Kirsteen and Alberto, 2014).** Furthermore, the metabolite plays a very important role in controlling the mechanisms of death, as well as in activating the survival pathways of brain cells **(Hall and Garthwaite, 2009).** Physiological concentrations of NO (100 pM –5 nM) have been shown to have a neuroprotective effect while high concentrations are on the contrary neurotoxic **(Calabrese *et al.*, 2007).** Interestingly, as early as 1982, NO was implicated in the immuno-defense network, as marked increase excretion was observed in human subjects with diarrhea and fever **(Wagner and Young, 1984).**

The effects of NO in the immune system are diverse. NO can have antiviral, antimicrobial, immunostimulatory, immunosuppressive, cytotoxic and cytoprotective effects. The production of NO appears to be necessary for the proper functioning of lymphocytes (NK) and considerably influences the production of cytokines involved in innate immunity **(Bogdan *et al.*, 2000).** Several studies show that NO can inhibit the rolling, adhesion and transmigration of monocytes and granulocytic leukocytes as well as the function or expression of certain neutrophilic integrins (**Grisham *et al.*, 1998)**. On the other hand, it has been shown that NO can have anti-cancer effects, in particular by preventing the progression of metastases and by promoting the regression of several tumours **(Bogdan, 2000).**

NO activates guanylate cyclase in vascular SMC and leads to increased cyclic guanosine monophosphate (cGMP), activation of cGMP mediated protein kinase G, and vasodilation **(Lloyd-Jones and Bloch, 1996)** NO has anti-atherogenic effects in addition to vascular tone adjustment capability **(Rafieian-Kopaei *et al.*, 2014).** In other cells such as immune cells or neurons, NO is produced by the inducible NO synthase (iNOS) and neuronal NO synthase (nNOS), and mediates an inflammatory response or acts as an atypical neurotransmitter, respectively. Furthermore, it was suggested that NO plays an important role in the regulation of energy balance (**Morley and Flood, 1994).**

The purpose of this topic is to high light studies relating to nitric oxide production. Moreover, to elucidate its roles nitric oxide as major preventing and regulating factor for modern diseases.

1. NO formation:

NO is synthesized from L-arginine in a reaction catalyzed by a family of nitric oxide synthase (NOS) enzymes. Active NOS is a tetramer formed by two NOS proteins and two calmodulin molecules. Conversion of L-arginine to NO and L-citrulline requires NADPH and O2 **(Korhonen *et al.*, 2005).** With the intermediate formation of N-hydroxyl- l-arginine (l-NOHA) (Figure 2) **(Boucher *et al.*, 1994)** as cosubstrates and (6R)-tetrahydrobiopterin (BH4), FAD, FMN and iron protoporphyrin IX (haem) as cofactors (figure1) **(Korhonen *et al.*, 2005).** This free radical has a biological half-life of seconds under normal physiologic oxygen tension **(Polat *et al.*, 2002).** It is produced by the endothelium, neurons, hepatocytes, neutrophils and macrophages **(Ischiropoulos *et al.*, 1992)**.

NO has multiple biological properties, in fact, it is a radical compound. The detector occurs in normal temperature conditions and pressure in an invasive form, its solubility in water is comparable to those in carbon monoxide (CO) and molecular oxygen (O_2). It is also soluble in non-polar solvents, making it easy to publish it through cell membranes **(Sennequier *et al.*, 1998).** Free radical nitric oxide can participate in a range of biochemical reactions, a strong biological oxidizer, where the signal transfer procedure is converted to oxidation processes **(Radi, 2019).** Through its accelerated interactions with superoxide (O_2-) for a pro-peroxentrate dose (ONOO-) **(Nagy *et al.*, 2007)** highly reactive and oxidizing molecule capable of affecting the functioning of several proteins **(Ischiropoulos *et al.*, 1992).**

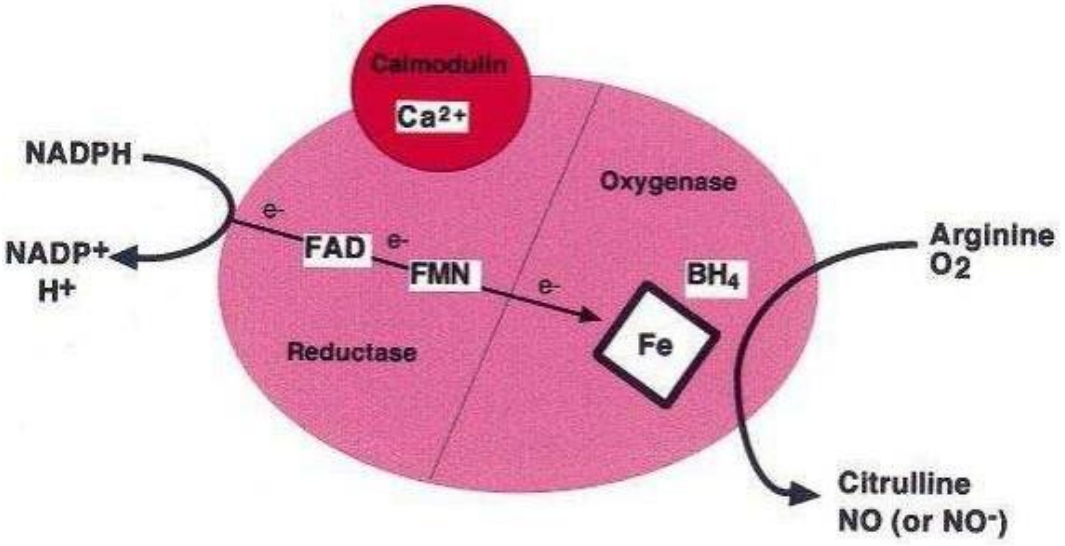

Figure 1. Overall reaction catalysed and cofactors of NOS **(Alderton *et al.*, 2001).**

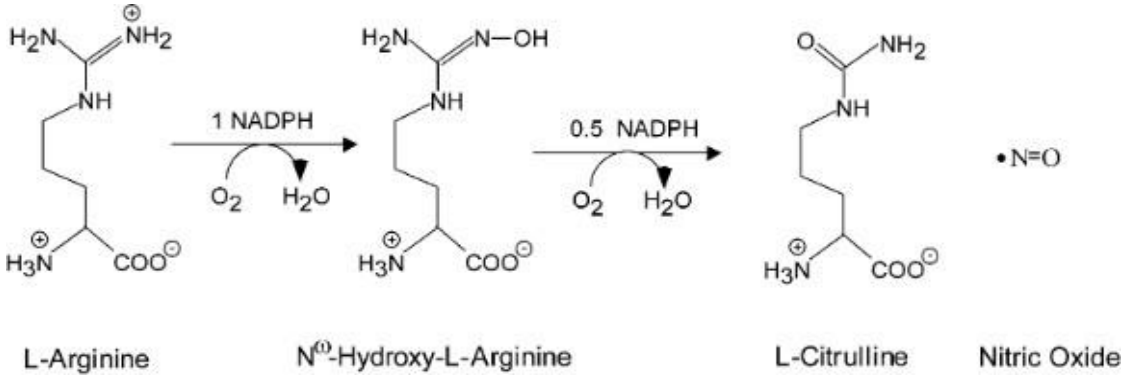

Figure 2. Biosynthesis of Nitric oxide **(Wei *et al.*, 2003).**

2. L-Arginine:

L-Arginine (Arg; freebase) is a naturally occurring amino Acid (AA) in animals and plants **(Rogers *et al.*, 1972)**. Hedin discovered its occurrence in mammalian protein in 1895 **(Gad, 2010).** It is a semi-essential or conditionally essential amino acid in humans is one of the most metabolically **(Morris, 2006).** In addition to its role in the manufacture of nitric oxide, arginine acts as an indicator for the synthesis of polyamines, proline, glutamate, creatine, agmatine, and urea. Several studies conducted on human and experimental animals have indicated that taking L-arginine has multiple beneficial pharmacological effects when taken in larger doses than normal food consumption **(Gad, 2010).**

The endogenous arginine pool is mainly derived from proteolysis. However, arginine biosynthesis is also possible from the carbon skeletons of glutamine or from the enzymatic recycling of citrulline **(Guelzim, 2011).** It is used across multiple pathways for protein synthesis and low molecular weight biologically active substances. Moreover, L-Arginine regulates cellular gene pathways Expression enhance lean tissue mass, reduce human obesity **(Rogers *et al.*, 1972)** and improving endothelial function and improving athletic performance from insulin resistance and improving rectal function. However, relatively large doses of L-arginine are required to produce these effects. Approximately 40% of L-arginine is metabolized orally by L-arginase in the first pass and 15% of L-arginine system is extracted and metabolized by the liver **(Suzuki *et al.*, 2016).** The nitric oxide is synthesized in the brain, especially in the kidneys from citrulline, due to the presence of the main synthesis of arginineoxinate and low activity of low arginine in the kidneys, since cytolin is mainly derived from intestinal metabolism of glutamine. It has been shown that, there is a direct relationship between the biological availability of glutamine and the ability of the intestine to produce citrulline and the synthesis of renal arginine **(Gulhermet, 1996).**

Arginine supplementation can sometimes lead to a significant increase in the plasma content of this amino acid. It is accompanied by a marked increase in the concentrations of ornithine and urea **(Rogers *et al.*, 1972)**. This is due to subverting arginine, such asstrengthening arginine, arginine to ornithine (figure 3) **(Morris, 2004).**

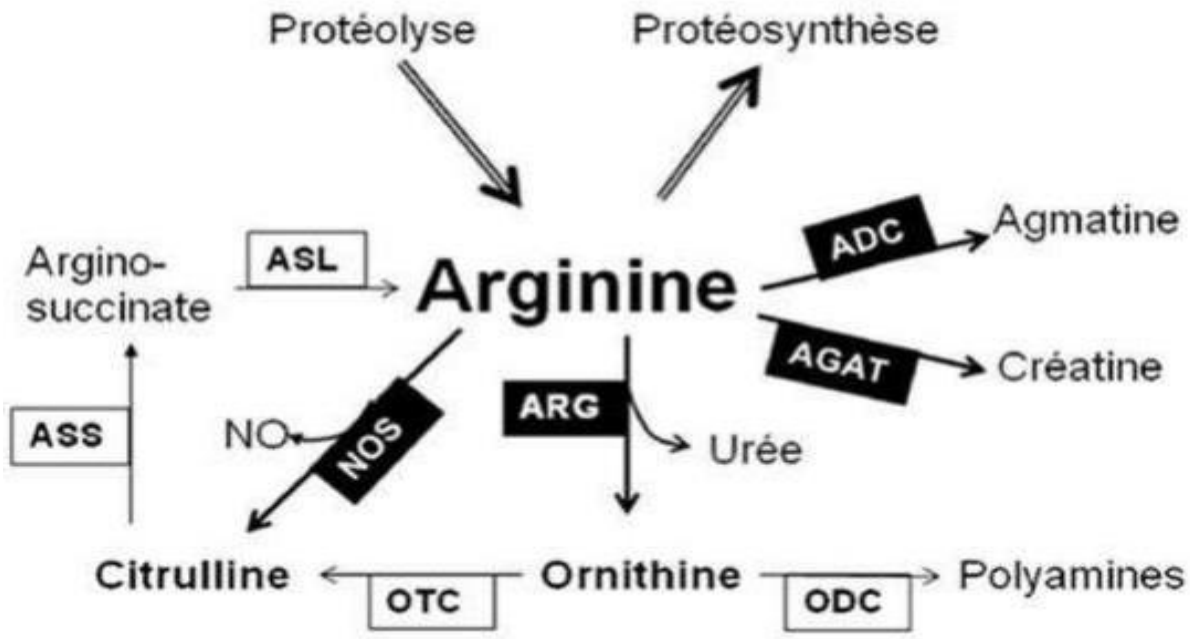

Figure 3. Routes of synthesis and use of arginine **(Morris, 2004).**

(ADC: Arginine Decarboxylase; AGAT: Arginine Glycine Amidinotransferase; ARG : Arginase; ASL: Argininosuccinate Lyase; ASS: Argininosuccinate Synthetase; ODC: Ornithine Decarboxylase; OTC: Ornithine Transcarbamylase) based on **(Morris, 2004).**

2.1. Association between nitric oxide and L-arginine:

In 1987, it had become clear that the generation of nitric oxide was not only occurring in the vascular wall. Potential formation of nitric oxide in macrophages. In addition, contain a low-molecular-weight stimulus from a soluble guanylate solution, which they later identified as L-arginine **(Moncada andHiggs., 2006).** However, its physiological function is not yet fully understood. However, current interest in L-arginine is focused mainly on its close relationship with the important signal molecule nitric oxide (NO), L-Arginine is the only substrate in the biosynthesis of NO, which plays critical roles in diverse physiological processes in the human body including neurotransmission, vasorelaxation, cytotoxicity and immunity **(Gad, 2010).**

The association of l-arginine intakes and systemic NO production is currently an important gap of knowledge with controversial data. Modulation of NO-related pathways following oral l-arginine supplementation has led to the current dominant belief that oral arginine intake does dependently modulate arginine bioavailability and promotes NO synthesis (**Loscalzo, 2000)** on the other hand, this relation is doubtful because NOS enzyme should theoretically be saturated in the presence of physiological concentrations of l-arginine (**Bode-Boger *et al.*, 2007).**

There is evidence to support absolute nutritional deficiency as a cause of vascular dysfunction in humans. Nevertheless, evidence has been provided supporting the importance of external supply of arginine to a healthy vascular system **(Kamada *et al.*, 2001).** As the endothelial vascular function was examined in a patient with lysinuric protein intolerance (LPI) who had a genetic defect in the transport of dibasic amino acids caused by mutations. The carrier is usually expressed in the intestinal and renal epithelial cells, and the lack of expression leads to impairment. Dietary absorption of exogenous arginine and impaired renal tubular absorption of filtered arginine. As a result, the patient's plasma-arginine concentration was significantly lower than normal (decreased by 79%) **(Gad, 2010).** The researchers revealed NO-dependent endothelial function in this patient with serum levels of nitric oxides (NOx) and a flow-mediated brachial vasodilator response approximately 70% lower than in controls. The patient also experienced decreased platelet counts, and increased plasma levels in contrast to the intravenous infusion of arginine all of these effects. The extracellular supply of L-arginine is essential for proper endothelial nitric oxide (eNOS) synthesis, despite the fact that intracellular arginine may well exceed a kilometer for eNOS; a phenomenon the literature calls the allergic nine paradoxes. Most researchers believe this phenomenon is caused by the admixture of cationic transporter arginine (CAT-1) with membrane-bound eNOS in plasma caves (**Kone *et al.*, 2003).**

3. Food Containing L-Arginine:

It is recommended that you eat adequate amounts of fruits and vegetables as part of a healthy diet. Fruits and vegetables may reduce chronic disease and, more specifically, coronary heart disease. These nutrients work through a variety of mechanisms, such as reducing stress with antioxidants, improving your lipoprotein profile, and lowering blood pressure. However, the recommendation to eat fruits and vegetables for the prevention of chronic diseases is mainly based on observational epidemiological studies, which leaves much uncertainty regarding the causal mechanism of this relationship **(Dauchet *et al.*, 2006).**

This may be because vegetables and fruits rich in nitrate can provide a physiological substrate for reduction to form nitrite and nitric oxide (NO). The beneficial effects of these foods

on the diseases resulting from circulatory disturbances are attributed to the cyclic guanosine monophosphate (cGMP)-dependent actions of NO, including vasodilation and vascular endothelial protection from platelet aggregation and leukocyte adhesion **(Davignon *et al.*, 2004).** However, in addition to these classical functions of NO, there indicated novel functions for NO through cGMP-independent and protein S-nitrosylation-dependent intracellular signaling pathways **(Hess *et al.*, 2005**). S-nitrosylation is associated with the activation of transcription factors, the regulation of a number of signal transduction molecules **(Bryan *et al.*, 2005**) and redox protein modification **(West *et al.*, 2006)** mitochondrial functions (**Larsen *et al.*, 2011),** and cell apoptosis **(Melino *et a*l., 1997).** In addition to endogenous NO generation through the l-arginine-NO synthase (NOS) pathway, NO is also generated through the independent NOS-nitrate, nitrite, NO pathway **(Weitzberg and Lundberg, 1998).** The various foods containing L-Arginine are Green leafy vegetables like lettuce, spinach, and beetroot containing high concentrations of nitrates and so on beets, spinach, radish, celery, cabbage, mushrooms, broccoli, green beans, strawberries, bananas, and green peppers **(Sindelar and Milkowski, 2012).** Green mustard, salad mix, coleslaw, tomato, vegetable soup, hot dog, bacon, banana, french fries, pork, fruit mix, orange, apple sauce, ketchup, carrots **(Hord *et al.* 2009),** Pomegranate juice (PJ) **(Ignarro *et al.*, 2006),** Citrus **(Yoshigai *et al.*, 2013),** Watermelon **(Mee *et al.*, 2018).**

4. Inhibitors of NO Syntase:

Competitive inhibitors of NO synthase are widely used **(Fukui and Chaudhuri, 1995)**. These include: NG-nitro-l-arginine (L-NNA), methyl ester of L-NNA (L-NAME) **(Marletta, 1994),** Nω-methyl-l-arginine (l-NMA) **(Ueno *et al.*, 1992),** also referred to as N^G-monomethyl-L-arginine (L-NMMA), occurs naturally in living organisms, as it is a product of the degradation of arginine-methylated proteins **(Bedford and Clarke, 2009**), Homothiocitrulline and S-methylthiocitrullin **(Narayanan and Griffith, 1994).** Nω-propyl-L-arginine **(Zhang *et al.*, 1997).**

The non-competitive inhibitors with L-arginine such as asymmetric dimethyl arginine (ADMA) **(Boger *et al.*, 2000)**, Opioids **(Kampa*et al.*, 2001),** mono amino acids **(Hunter and Downs, 1944).**

Asymmetric dimethyl arginine (ADMA) is a non-competitive inhibitor of an isolated nNOS **(Boger *et al.*, 2000).** ADMA is a more potent inhibitor of nNOS than NMA **(Tsikas *et al.*, 2000).** In various pathological conditions associated with endothelial dysfunction such as essential hypertension, atherosclerosis and hypercholesterolemia, ADMA plasma concentrations are elevated while urinary excretion of the NO metabolites nitrite and nitrate and of cGMP, the second messenger of NO, are reduced both in animals and in humans; under these conditions, the L-

arginine concentration is almost normal **(Böger *et al.*, 1997).** ADMA inhibits the production of vascular NO at concentrations present in pathophysiological conditions; ADMA also causes localized vasoconstriction when it is implanted into an artery. Thus, elevated ADMA levels may explain the "L-arginine paradox", the observation that supplementation with exogenous L-arginine improves vascular function mediating NO in vivo. Dimethyl arginine is the result of the degradation of methylated proteins; the methyl group is derived from S-adenosylmethionine. ADMA, the homologous dimethyl arginine, is eliminated from the body by renal excretion; Whereas ADMA is only metabolized by hydrolysis to citrulline and dimethylamine by the enzyme dimethyl arginine dimethyl amino hydrolase (DDAH). Thus, the activity of its expression DDAH may contribute to the pathogenesis of endothelial dysfunction in various diseases. Increased levels of ADMA correlated with decreased NO synthesis as assessed by endothelial dependent vasodilation. ADMA has become a target of pharmacotherapeutic interventions. Among other potential strategies currently being tested, administration of L-arginine has been shown to improve endothelial-dependent vascular function in people with high levels of ADMA **(Rainer, 2004).**

Arginine analogues the majority of investigations into the inhibition of nNOS are related to design and evaluation of reversible inhibitors that mimic either l-arginine. These inhibitors are designed to simply outcompete L-arginine and bind in the enzyme's active site, thus preventing arginine turnover. Early investigations (1990–1995) were focused on the development of inhibitors derived from arginine it self, and one of the first nNOS inhibitors discovered was N^{G}-nitro-l-arginine (L-NNA) **(Marletta, 1994).** This compound is a potent nNOS inhibitor (**Furfine *et al.*, 1993)** that undergoes little metabolism and rapidly penetrates the mouse brain (**Bansinath *et al.*, 1993).** The methyl ester of L-NA, L-NAME, is more potent, possibly because of increased bioavailability **(Marletta, 1994).** Nonetheless, these simple nitroarginines are nonselective NOS inhibitors, and while 1 has around 300-fold selectivity in favor of nNOS over iNOS (**Raman *et al.*, 1998),** nitroarginines cause severe hypertension when administered to animals as a result of potent inhibition of eNOS as well (**Kobayashi *et al.*, 1991).**

Many modifications made to L-arginine at positions other than the guanidine moiety (e.g., introduction of aromatic groups, sulfonic acids, and heterocycles) resulted in compounds that are neither NO inhibitors nor substrates **(Yokoi *et al.*, 1996),** but many arginine derivatives alkylated on the guanidine group possess NOS inhibitory activity. Hibbs and collaborators (1987) first described N^{ω}-methyl-l-arginine (l-NMA) as a NOS inhibitor. Although l-NMA was shown to accumulate in the brains of rats and cattle **(Ueno *et al.*,1992),** and showed some ability to attenuate migraine headaches in human clinical trials **(Lassen *et al.*, 1998),** this compound also has adverse cardiovascular effects in both animals and humans, because of nonselective NOS

inhibition **(Lopez*et al.*, 2004).** Similarly, higher alkylarginines (containing dimethyl, cyclopropyl, etc.) are also inhibitors of multiple NOS isoforms **(Marletta, 1994).**

Calmodulin Antagonists like inhibitors that bind at the active site of NOS and additionally interact with H_4B, imidazole-based inhibitors are known that bind competitively at heme and interact with the calmodulin (CaM) binding site. Miconazole, ketoconazole, and clotrimazole are imidazole-containing antifungal agents that showed competitive inhibition of l-citrulline formation in bovine nNOS, while they also showed calmodulin-dependent competitive inhibition of cytochrome reductase activity of nNOS **(Wolff *et al.*, 1993).** Although these compounds show selectivity over iNOS, there was no selectivity between nNOS and eNOS **(Wolff and Gribin 1994).**

Melatonin and kynurenine derivatives are metabolites of tryptophan in the brain and possess neuroprotective properties **(Reiter *et al.*, 1995).** Melatonin exhibits Ca^{2+}-dependent rat nNOS inhibition with an IC_{50} of 0.1 µM. Additionally, a significant inhibition of rat nNOS (>22%) was observed at 1 nM melatonin concentration, which is its physiological serum concentration at night **(Pozo *et al.*, 1994).** Melatonin and these synthetic kynurenine derivatives modulate the excitatory response of NMDA-receptors in rat striatal neurons, an effect that is related downstream to nNOS inhibition **(León *et al.*, 1998). s**tudies on a series of synthetic kynurenines revealed that an amino substitution on the benzene ring is crucial and provided maximal nNOS inhibitory activity in the series (**80**, IC_{50} = 40.9 µM) **(Camacho *et al.*, 2002).** The inhibitory effects of melatonin and amino-kynurenines were shown in kinetic studies to be dependent on CaM concentration, which suggests that CaM may be involved in the intracellular neuroprotective effect **(León et *al.*, 2000).** Trifluoperazine, an antipsychotic agent with antimodulin activity, inhibits the release of catecholamine from cultured chromaffin cells at a step further away from calcium entry and therefore, has a role for calmodulin in the secretory process of these cells **(Kenigsberg *et al.*, 1982).**

5. NO synthase isoenzymes:

The differences in properties and differential inhibitor substrate specificities suggest that there are several forms of NO synthase. Three distinct NO synthase has been confirmed **(Alderton *et al.*, 2001):**

5.1. Endothelial nitric oxide synthase (eNOS):

Endothelial nitric oxide synthase (eNOS) has an approximate molecular weight of 133 kDa. This enzyme is expressed mainly in endothelial cells but it has also been found in

cardiomyocytes, human placental syncitiotrophoblasts, LLC-PK1 renal tubular epithelial cells (pig kidney epithelial cells) and certain neurons **(Forstermann *et al.*, 1994).** In cells, it is associated with the plasma membrane and the membrane of the Golgi apparatus. Incorporation of myristic and palmitic acids at the N-terminus promotes the anchoring and interaction of the enzyme with membrane phospholipids. Activation of CaM by Ca^{2+} is essential for the enzyme to be functional. Thus, eNOS synthesizes NO in a pulsatile fashion when intracellular Ca^{2+} increases and induces CaM binding to the enzyme **(Hemmens and Mayer, 1997)**. However, other proteins can interact with eNOS and affect its activity. Heat shock protein 90 (hsp90) has been shown to promote allosteric activation of the enzyme as well as its coupling **(Pritchard *et al.*, 2001).** Caveolin-1 may also interact with and inhibit eNOS. Therefore, mice genetically invalidated for calveolin-1 have been shown to exhibit better endothelium-dependent relaxation **(Drab *et al.*, 2001)**. Furthermore, it has been shown that eNOS could be activated by mechanisms independent of the concentration of Ca^{2+} such as shear stress. Shear stress through activation of the PI3K/Akt pathway has been demonstrated to promote serine phosphorylation (Ser1177) of eNOS, which in turn increases the activity of the enzyme **(Dimmeler *et al.*, 1999).**

The NO produced by eNOS plays a very important role in the regulation of blood pressure. The vasodilatory effect of NO gives it the ability to counteract vasoconstriction controlled by the renin-angiotensin-aldosterone system **(Forstermann *et al.*, 1994).** Endothelial NO is a key mechanism for maintaining vascular homeostasis not only through its vasodilator effects but also through its anti-atherogenic properties. It has been shown that endothelial NO can inhibit platelet aggregation, the expression of certain chemokines, including MCP-1 and leukocyte adhesion to vascular walls by interfering with the expression or activity of CD 11 integrin. /CD 18 **(Zeiher *et al.*, 1995).** It also participates in the remodeling of the vascular wall by preventing the migration and proliferation of smooth muscle cells **(Garg and Hassid, 1989).** On the other hand, the NO produced by eNOS inhibits endothelial cell apoptosis induced by radical oxygen species (ROS) and angiotensin II **(Dimmeler and Zeiher, 1999).**

Mice genetically disabled for eNOS exhibit a failure in the mechanisms of NO-dependent vasodilation as well as arterial hypertension **(Huang *et al.*, 1995).** In models of cerebral ischemia and vascular trauma, these mice were more susceptible to stroke as well as increased neointimal proliferation, respectively **(Moroi *et al.*, 1998).** Furthermore, these mice have been observed to exhibit increased left ventricular contractility in response to β-adrenergic agonists as well as pulmonary hypertension **(Gödecke *et al.*, 2001).** However, heterozygous mice normally

express a functional eNOS gene in basal state while unable to modulate their vascular reactivity during physical activity **(Kojda *et al.*, 2001).**

Obesity can affect eNOS expression levels. The expression of this enzyme has decreased in the skeletal muscle and adipose tissue of rodents and obese subjects **(Kraus *et al.*, 2012).** Furthermore, the expression of an eNOS negative regulator, caveolin-1, is increased in the aorta of obese rats and that ceramides can inhibit the activity of the enzyme by dissociating the complex. Formed between eNOS-Akt and hsp90 **(Zhang *et al.*, 2012).** Phosphorylation of eNOS on serine residue 1177 is reduced in mice subjected to a diet rich in lipids **(Kim *et al.*, 2008).** Studies showed that endothelial insulin resistance decreases the bioavailability of NO and contributes to the development of endothelial dysfunction **(Duncan *et al.*, 2008).** Furthermore, the decrease in eNOS phosphorylation observed during insulin resistance appears to be partly responsible for the decrease in muscle glucose uptake in mice fed a diet rich in lipids **(Kubota *et al.*, 2011).**

5.2. Neuronal nitric oxide synthase (nNOS):

Neuronal nitric oxide synthase (nNOS) has a molecular weight of approximately 160 kDa. This enzyme is expressed in mature and immature neurons as well as in astrocytes, cardiomyocytes, gastrointestinal smooth muscle, keratinocytes, macula densa, skeletal muscle, vascular smooth muscle cells and hepatocytes among others **(Villanueva and Giulivi, 2010).** The activity of nNOS is controlled by CaM and the intracellular concentration of Ca^{2+}. In the brain, nNOS is found in particles or in soluble form and it seems that its physiological functions depend on its subcellular location. This enzyme has a PDZ domain (PSD95, DLG, ZO1) that can interact with that of several proteins, and thus affect the subcellular distribution or activity of the enzyme in the brain and muscle **(Zhou and Zhu, 2009).**

The phosphorylation of nNOS at different sites has been observed to regulate the activity of the enzyme. Calmodulin-dependent protein kinase II (CaMKII) protein kinase phosphorylates nNOS on serine residue 847 (Ser847) and inhibits Ca^{2+} binding to CaM, while dephosphorylation of this site by phosphatase 1 (PP1) results in increased activity of the enzyme **(Rameau *et al.*, 2004).** There is also an nNOS phosphorylation site on serine residue 1412 (Ser1412) analogous to that of Akt present in eNOS **(Adak *et al.*, 2001).** Phosphorylation at this site promotes electron flow from the reductase domain of nNOS and increases the sensitivity of the enzyme to the Ca^{2+}/CaM complex **(Rameau *et al.*, 2007).** Phosphorylation on serine residue 741 (Ser741) of nNOS by the CaMKI calmodulin-dependent protein kinase I) has also been shown to inhibit the activity of the enzyme in vitro **(Song *et al.*, 2004).**

The NO produced by nNOS plays important roles in learning, memory and neurogenesis. In the central nervous system, nNOS regulates long-term potentiation and thus contributes to the consolidation of memory in the hippocampus **(Bon and Garthwaite, 2003).** Several studies show that inhibition of nNOS can affect memory and learning processes, while others have found no significant effect on the latter **(Bannerman *et al.*, 1994).** There are also studies that show the involvement of NO produced by nNOS in the central regulation of blood pressure **(Togashi *et al.*, 1992).** Therefore, inhibition of the activity of this enzyme in the medulla oblongata and hypothalamus has been demonstrated to promote the development of hypertension **(Toda *et al.*, 2009).** On the periphery, the NO derived from nNOS can act as a neurotransmitter on nitrergic neurons by stimulating the NO-sensitive guanylate cyclase of its target cells, which causes the decrease in tone of several types of smooth muscle cells including those of the blood vessels Blood **(Forstermann *et al.*, 1994).**

Defects in NO signaling in the nervous system contribute to the development of several neurodegenerative diseases. Several studies show a correlation between the excessive production of NO, or its derivatives, and pathologies such as multiple sclerosis, Alzheimer's and Parkinson's **(Steinert *et al.*, 2010).** NNOS has been shown to be overexpressed in basal ganglia as well as in neutrophils of patients with Parkinson's **(Gatto *et al.*, 2000).** Mice geneticallydisabled for nNOS are more resistant to the effects of MPTP (1-methyl 4-phenyl 1, 2, 3, 6-tetrahydropyridine), a neurotoxin commonly used to study Parkinson's disease **(Matthews *et al.*, 1997).** Activation of nNOS is associated with the development of lesions following ischemic stroke **(Eliasson *et al.*, 1999).** Mice genetically invalidated for nNOS show a reduction in the size of the cerebral infarction compared to control mice in models of cerebral ischemia **(Huang, 1999).**

5.3. Inducible nitric oxide synthase (iNOS):

Inducible nitric oxide synthase (iNOS) is a protein with a molecular weight of approximately 131 kDa. The enzymatic activity of iNOS is not dependent on intracellular Ca^{2+} concentrations. This protein can be induced in several cell types by different inflammatory stimuli and its activation triggers a sustained production of NO, which is maintained until the enzyme is degraded **(MacMicking *et al.*, 1996).** Unlike constituent NOS, iNOS can produce large amounts of NO for long periods of time **(Pautz *et al.*, 2010).** NO produced by iNOS may have protective effects due to the antimicrobial, antiviral, antiparasitic and antitumor properties of NO. On the other hand, an aberrant induction of this enzyme can have harmful consequences for the adequate functioning of several systems. Induction of iNOS has been shown to be associated with the

development of several pathologies including asthma, arthritis, multiple sclerosis, metabolic and neurodegenerative diseases, and cancer **(Kroncke *et al.*, 1998).**

The expression of iNOS is regulated by the presence of cytokines or other inflammatory stimuli such as LPS. Thus, iNOS expression depends primarily on de novo synthesis and the stability of mRNA and protein **(Carpenter *et al.*, 2001).** In fact, it has been observed, in different cellular models, that the interaction of several inflammatory stimuli could have synergistic effects on the induction of iNOS at the transcriptional level **(Nussler and Billiar, 1993).** It has also been shown that, iNOS could be induced by factors that are found to be increased in obesity such as ceramides, hyperglycemia, GLA and CRP, among others **(Won *et al.*, 2004).** iNOS gene requires the participation of several transcription factors. Among the transcription factors involved in the activation of iNOS are NF-κB, AP-1 (activator protein 1), STAT-1 α (signal transducer and activator of transcription), IRF-1 (interferon regulatory factor-1), NF -IL-6 (nuclear factor interleukin-6) and HMG-I/Y (high mobility group proteins I/Y) **(Ganster *et al.*, 2001).** Given the variability of the inflammatory stimuli that can lead to the activation of iNOS, several intracellular signaling cascades, including the Jak (Janus kinases), MAPK (mitogen-activated protein kinases), Raf-301 (serine/threonine-protein kinase proto-oncogene), and PP (protein phosphatases), promote expression of the enzyme **(Bogdan, 2001).** The transcription of iNOS can also be controlled by the levels of NO. Low concentrations of NO promote the activation of NF-κB and therefore the expression of iNOS. On the other hand, it appears that high concentrations of NO inhibit the induction of the enzyme in order to avoid overproduction of NO **(Connelly *et al.*, 2001).**

The activity of iNOS may be affected by the availability of its substrate and its cofactors. **(Chang *et al.*, 1997)** demonstrated that the production of NO by macrophages depends on the concentration of extracellular arginine, independent of intracellular levels of the latter**.** The extracellular concentration of arginine depends on the activity of arginase, the enzyme responsible for breaking down this amino acid into urea and ornithine. When arginase expression increases before iNOS induction is started, NO production is significantly affected. However, when these two enzymes are induced simultaneously, NO production appears to be affected, given the higher affinity of iNOS for its substrate **(Wu and Morris., 1998).** The transport of arginine to the interior of cells takes place using CAT proteins (cationic amino acid transporter proteins). It would also seem that the activity of iNOS is affected by the transport of arginine because it has been observed that macrophages stimulated with LPS and IFN-γ, from mice genetically invalidated for CAT2, produce very little NO **(Nicholson *et al.*, 2001).** On the other hand, the activity of iNOS also

depends on the mechanisms regulating the synthesis and availability of BH4, since it is an essential cofactor for its homodimerization **(Leonova *et al.*, 2008).** Furthermore, it seems that the phosphorylation of iNOS could affect its activity. The phosphorylation of iNOS has been shown to correlate with its activity in murine macrophages treated with LPS **(Salh *et al.*, 1998).**

6. Regulation of NO Synthase:

The effective formation of NO is only possible if the NOS are active. Now the biosynthesis of NO is regulated at several levels: transcriptional, but also by the concentration of the medium in calcium ions, by the assembly of the dimer of NOS, or by their substrate and product (arginine, NO) where NOS-1 (nNOS) and NOS-3 (eNOS) are constitutive and NOS-2 (iNOS) inducible. NOS-2 is indeed expressed in most mammalian nucleated cells examined, under the action of endotoxins (lipopolysaccharides) or cytokines (TNF-α, tumor necrosis factor α, interleukins, and interferonγ) via activation of transcription factors such as NF-κB. This relates in particular to cells of the immune system such as macrophages **(Thiemermann, 1995),** including humans. The difficulty in highlighting the expression of NOS-2, which has been observed with human macrophages, seems to be raised when the macrophages come from patients suffering from various infectious or inflammatory diseases. NOS-2 is not, however, uniquely inducible, because it is continuously expressed in human respiratory epithelium, blood platelets and, transiently, in cerebral blood vessels during rat brain development. Conversely, there is some regulation of the expression of NOS- 3 by factors such as shear stresses or estrogen exposure. The distinction between constitutive and inducible isoforms extends to regulation by calmodulin (CaM) coupling. NOS-1 and 3 are in fact active only after coupling to calmodulin, which intervenes as a switch allowing the flow of electrons within the enzyme **(Abu-Soud *et al.*, 1994).**

The activity of these two isoforms is therefore regulated by the concentration of the medium in calcium ions which regulate the coupling of CaM **(Thiemermann, 1995).** The activity of NOS-2 is, on the other hand, independent of calcium ions, because this isoform is strongly coupled to calmodulin. As a result, NOS-2 continuously produces NO. The non-regulation of NOS-2 by calcium lesions makes adequate expression of this isoform all the more important. Calmodulin binding is also thought to be involved in the assembly of NOS-3 monomers **(Hellermann and Solomonson, 1997).** Dimerization could therefore constitute another means of regulating NOS. An endogenous inhibitor of NOS-1 has recently been highlighted, PIN (inhibitory protein of NOS-1), one of the most conserved proteins in nature **(Jaffrey and Snyder., 1996).** It probably acts by destabilizing the dimer phosphorylation could represent -sent an additional

regulatory mechanism for the set of NOS, but the role of this post-transcriptional modification is not yet well understood **(Michel and Feron, 1997).**

The substrate itself plays a role in the regulation of the activity of the NOS. In fact, like some P-450 cytochromes, the NOS produce, in the absence of the substrate, superoxide ions and hydrogen peroxide, by transfer of electrons from NADPH to O2 by vialheme (transfer called "decoupling"). L-arginine saturation causes a decrease in the rate of oxidation of NADPH For NOS-1, this decrease probably results from regulation by the calcium concentration, which suggests that, since the substrate is not lacking normal operating conditions, uncoupling rarely occurs. For NOS-2, the decrease in the consumption of NADPH when the substrate runs out could be a mechanism limiting the production of oxidizing species if the activity of this isoform were to be regulated by the concentration of L-arginine. Substrate could regulate the activity of NOS. The arginine available, and therefore the production of NO, could be modulated by arginase **(Chang *et al.*, 1998).** An enzyme which transforms arginine into urea and ornithine, and is present (among others) in endothelial cells and macrophages. In addition, N-omega-hydroxy-L-arginine (NOHA) has recently been shown to be one of the best arginase inhibitors **(Boucher *et al.*, 1994).** There may therefore be a cross-regulation of the two enzyme systems metabolizing arginine, NOS and arginase. It inhibits NOS: it can indeed attach itself to the iron atom of NOS, even before leaving the active site. The resulting inactive complex is formed transiently during the production of NO; it represents 80% of the total enzyme in the case of NOS-1. It is unstable in the presence of O2, the concentration of which modulates the activity of NOS-1. The NOS could therefore play the role of O2 probes **(Dweik *et al.*, 1998).**

7. Role of nitric oxide:

7.1. No and Endothelium-derived relaxing factor (EDRF):

In isolated blood vessels, the mechanical or enzymatic removal of the endothelium leads to increased contractions that are evoked by a variety of vasoconstrictor agents, because the endothelial cells release the powerful relaxing substance (s) (relaxing factors) derived from the endothelium (EDRF) **(Vanhoutte, 1989).**

Endothelium-derived relaxing factor (EDRF) is an endogenous vasodilator that endothelial cells produce and subsequently release in response to various changes in normal physiologic as well as pathophysiological changes. EDRF causes vascular smooth muscle to relax, and it is structurally in the form of nitric oxide (NO) or a compound that contains nitric oxide. Where EDRF is formed from L-arginine by an enzyme that is dependent on calcium-calmodulin and

NADPH and it serves as an inhibitor of aggregation and adhesion of platelets and is a vasodilator. EDRF also serves as a second messenger for guanylyl cyclase activation and cyclic GMP production **(Oliveira-Paula *et al.*, 2016)** for cells in cardiovascular tissue, respiratory and renal epithelium, macrophages, neurons of the cerebellum, and adrenocytes **(Paulo *et al.*, 2014).**

Nitric oxide (NO) is an unstable vasodilator independent of endothelial release from vasodilators such as nitroprusside and glyceryl trinitrate. The researchers have repeatedly observed that the actions of NO on vascular smooth muscle are very similar to those of EDRF and therefore, EDRF released from an artery and vein possess identical biological and chemical properties as NO **(Ignarro *et al.*, 1987).**

EDRF acts directly on the vascular smooth muscle to expand autoimmune vasodilation and inhibit sympathetic vasoconstriction **(Zanzinger *et al.*, 1994),** It also acts as a nitric oxide by activating the soluble guanylil cyclase. The endothelial cell membranes contain an enzyme called endothelial nitric oxide synthase that produces nitric oxide, which binds to the soluble guanylil ring. This binding triggers a chain of cellular signals, which ultimately leads to arterial vasodilation. The endogenous nitric oxide production process physiologically modifies blood flow and vascular movement **(Helms *et al.*, 2018).** It also has anti-platelet properties by inhibiting platelet adhesion and platelet aggregation. The decrease in platelet activation directly leads to an anticoagulant effect **(Gambaryan andTsikas. 2015).**

Nitric oxide has multiple functions **(Balligand *et al.*, 1993),** It is internally generated by compounds (nitric oxide synthase; NOS), as well as compounds containing NO and/or inorganic anions as nitrate and nitrite. (eNOS), critically involved in the maintenance of vasomotortone, and is sensitive to free calcium. Thus, upon increased intracellular calcium concentration and/or binding to the calcium/calodolin complex, eNOS is phosphorylated and NO is produced from L-arginine. The diffusion of nitric oxide into adjacent smooth muscle cells results in its binding to the ferric heme (Fe^{2+}) group of soluble Guanylyl Cyclase (sGC). This activated form of the sGC enzyme converts guanosine triphosphate (GTP) into a second cyclic monophosphate (cGMP) messenger, which reacts and activates cGMP-dependent protein kinases (PKGs). PKG induces activates smooth muscle relaxation by multiple mechanisms, including modulation of light chain mucin kinase, and reduces intracellular free calcium, as well as cell membrane hyperpolarization by regulating the activity of potassium channels or Na^{+}/K^{+} ATPase **(Michael *et al.*, 2014).** The lack of release in basal vascular tone contributes to data from eNOS knockout mice showing increased systemic resistance, blood pressure, and findings from humans and other experimental animals given NOS inhibitors such as L-NMMA **(Tsai and Kass, 2009).**

NG-monomethyl-L-arginine (L-NMMA), unlike noradrenaline or angiotensin, is devoid of any vasoconstrictor activity in the blood vessels. It is important in controlling and regulating blood flow and blood pressure. So the researchers suggested an effective vasodilating system, represented by the NO generation, which is constantly opposed by vasoconstrictor effects in the vessel wall **(Huang *et al.*, 1995).** There is indirect evidence that the nitric oxide system may be impaired in hypertensive patients. Some studies have measured blood flow in the forearm in patients with hypertension and hypertensive patients. The results indicate an abnormal expansion of basal nitric oxide in the forearm arterial bed of patients with untreated essential hypertension **(calver *et al.*, 1992).** Decoupling of eNOS resulting from reduced NO production, in an in vivo model of angiotensin II-induced hypertension, can occur due to deficiency of a cofactor in the substrate, or by direct biochemical disturbance of the enzyme. Depletion of the cofactor BH4 can be induced by oxidation of the BH3 and BH2 radicals, neither of which can support the generation of NO by NOS **(Tsai and Kass, 2009).**

In 1989 researchers found that if they injected L-NMMA, the NO synthase inhibitor, intravenously into an anesthetized animal there was along-lasting increase in blood pressure **(Moncada *et al.*, 1989).** This increase in blood pressure is accompanied by inhibition of NO Synthes in the vessel wall and can be immediately reversed by administering L-arginine, a specific inhibitor of endothelial-derived nitric oxide synthesis, in the brachial arteries of healthy volunteers to study the role of NO in controlling blood flow in the forearm. L-NMMA caused a 50% decrease in basal blood flow and moderated the extended response to the injected acetylcholine but not that to glyceryl trinitrate. These results indicate that the noninvasive action of endothelial-derived nitric oxide contributes to controlling basic and stimulating human blood flow. Impaired nitric oxide production may be responsible for the abnormalities in vascular reaction that characterize a variety of disease states. It also suggests that he could not be generated permanently inside or the fence, or was responsible for maintaining the enlarged vessel of the circuit **(Vallance *et al.*, 1989).** In another trial, male Brattleboro mice were given a solution of NG-monomethyl-L-arginine (L-NMMA; 1 mg ml-1) to drink for 7 days. There was a persistent rise in mean arterial blood pressure, accompanied by significant posterior vasoconstriction. Within 9 hours of the L-NMMA withdrawal, not all variants were different from pre-L-NMMA values. Brattleboro rats (n = 3) that were drinking the NG-nitro-L-arginine methyl ester (L-NAME) solution (0.05 mg mL-1) for 5–6 months showed a rise in blood pressure that reversed to normal within 48 hours. After pulling out L-NAME.Thus, inhibition of nitric oxide synthesis leads to long-term, but reversible, hypertension **(Gardiner *et al.*, 1992).**

Vascular endothelial cells play a key role in cardiovascular regulation by producing a number of potent vasoactive agents, including the vasodilator molecule nitric oxide (NO) and the vasoconstrictor peptide endothelin (ET). Most of the interventions trying to improve endothelial dysfunction have targeted one or more of the many risk factors that can cause damage to the lining of blood vessels: high blood pressure (ACE inhibitors and calcium antagonists), hypercholesterolemia (lipid-lowering agents), cigarette smoking (quitting) Inactivity, life style (increased physical activity), menopause (estrogen replacement therapy), and diabetes (control of metabolic abnormalities). Beneficial changes in the endothelium may result from enhancing vasodilation, inhibiting vasoconstriction, reducing production of free radicals, or other mechanisms that protect the endothelium from injury **(Pesić *et al.*, 2006).**

Nitric oxide (NO) plays an essential role in maintaining normal motor tone. There is a critical function of hemoglobin and erythrocytes in regulating NO activity in the vascular compartment. They may present NO as hemoglobin oxygen depressants. Intravascular hemolysis releases hemoglobin from red blood cells into plasma, which is then able to eliminate endothelial-derived NO faster than hemoglobin into RBCs. This may lead to vasoconstriction, decreased blood flow, activation of platelets, increased endothelial expression-1 (ET-1), and injury to the latter organ. Treatment with inhalation of NO gas or infusion of sodium nitrite during hemolysis may alleviate this disturbance of motor homeostasis. By oxidation of plasma cell-free hemoglobin **(Gladwin *et al.*, 2004).**

NO liberated from the lining of conductivity and resistance vessels is absorbed by red blood cells and inactivated by HbO2 by stoichiometric conversion to MetHb and nitrates **(Wennmalm *et al.*, 1992),** The nitric oxide (NO) concentration changes were monitored with a nitric oxide sensitive electrode in phosphate buffered saline (PBS) with free oxyhemoglobin (oxyHb) or erythrocytes. The half-life of nitrogen oxide is inversely proportional to the concentration of erythrocytes, regardless of the concentration of oxy-Hb inside RBCs, and the disappearance rate of nitric oxide is first order in nitric oxide concentration and first order in RBC concentration. After all oxy-Hb reacts with NO to form methemoglobin, the disappearance rate of NO slows down dramatically. These data indicate that the interaction of nitric oxide with oxyhemoglobin within RBCs is limited by diffusion of nitric oxide into the cell, which was also shown prior to the interaction of O2 with deoxyhemoglobin **(Liu *et al.*, 1998).** Moreover, S-nitrosylated or deoxyhemoglobin treated with nitrite may act as donors of NO instead of NO as scavengers. (Figure 4) **(Michael *et al.*, 2014).**

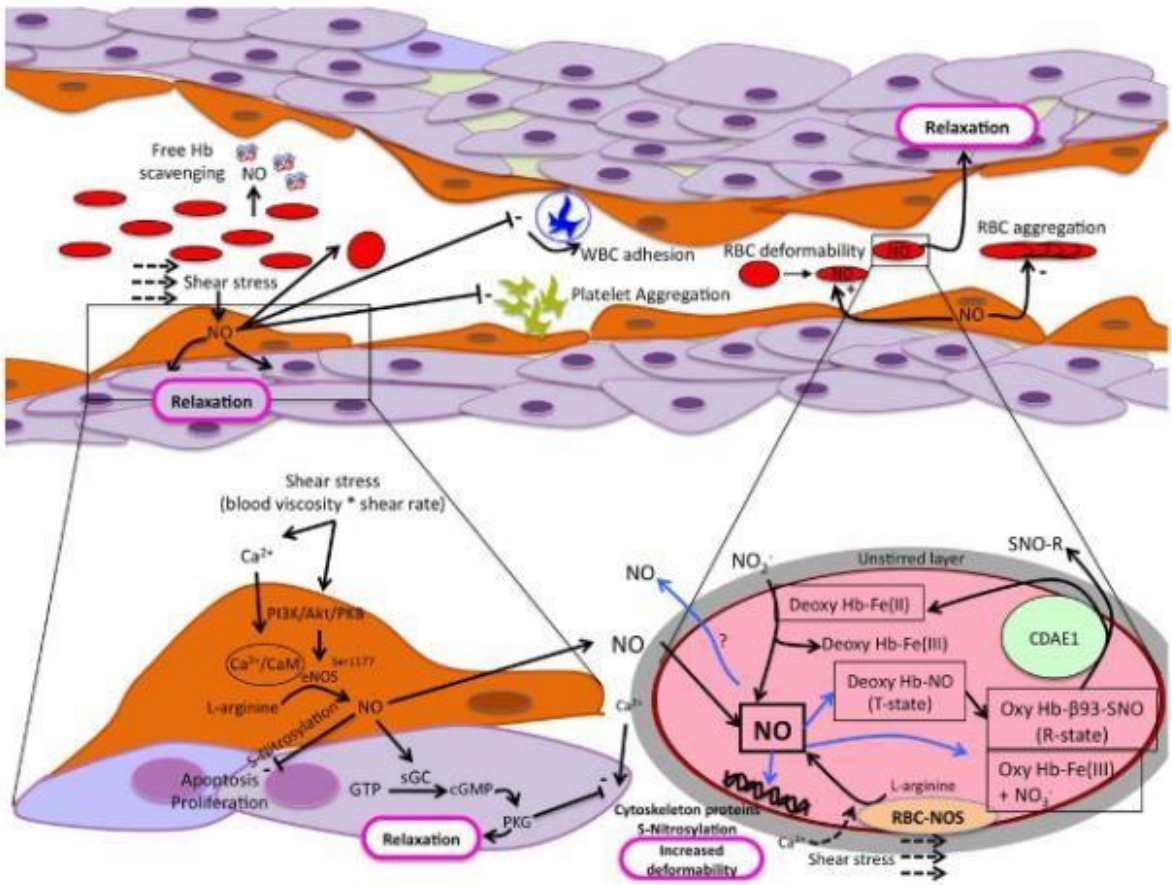

Figure 4. Nitric oxide (NO) generation pathways in endothelial cells and red blood cells (RBC) **(Michael *et al.*, 2014).**

7.2. No and Cancer:

As NO is a free radical, it is a highly reactive molecule within biological systems, capable of interaction with other free radicals, molecular oxygen and heavy metals. The biological effects of NO can be mediated by the products of different NO metabolites. For example, NO rapidly reacts intracellularly to form nitrite and nitrate, S-nitroso-thiols or peroxynitrate, and these metabolites are believed to play key roles in mediating many of the NO-associated genotoxic effects. These effects include DNA damage, which can be initiated by nitrosative deamination, DNA strand breakage or DNA modification **(Wink *et al.*, 1991).**

One of the consequences of the NO- mediated DNA damage is to trigger p53 accumulation, which can induce apoptosis. This is a possible process by which NO may induce death of tumour cells. An increase in NOS activity (arising from increased transcriptional activity, or from post-transcriptional/protein regulation activity) in tumour cells can consequently cause the

concentration of NO to be elevated such that it triggers p53-mediated growth arrest and apoptosis (**Ambs *et al*., 1997)**.

Interestingly, it has been demonstrated that accumulation of p53 results ultimately in down regulation of iNOS expression by inhibition of iNOS promoter activity **(Ambs *et al*., 1998).** Thus a negative feedback loop is formed between NO-generation and p53 accumulation that may constitute part of a physiological mechanism, which responds to endogenously produced DNA damage due to NO. Overall, this p53-mediated growth inhibition may be expected to provide a strong selection pressure for mutant p53 expression in tumor cells. In addition to p53, NO has also been shown to activate poly (ADP-ribose) polymerase (PARP) **(Zhang *et al*., 1994)** and it has been proposed that this activation is due to DNA damage. This damage may take the form of DNA strand breaks or nitrosative deamination of DNA bases when NO is generated at high concentrations. These high concentrations of NO have been reported for NMDA-mediated neurotoxicity as well as for tumouricidal and bactericidal activation of cells **(Wink *et al*., 1991)**.

Another important DNA repair enzyme, DNA-dependent protein kinase (DNA-PK), is also known to be essential for the maintenance of the structural integrity of the genome. DNA-PK is a serine/threonine protein kinase consisting of a large catalytic subunit (DNA-PKcs) and a regulatory subunit (Ku). Recently, mammalian DNA-PKcs has been shown to be an essential component of the DNA double-strand repair pathway, as well as being crucial for V(D) J recombination, involved in the generation of immunoglobulin and T-cell diversity. Scid mice, which lack DNA-PKcs, show increased susceptibility to ionizing radiation in addition to having impaired V(D) J recombination and arrested T- and B-cell development **(Smith and Jackson, 1999)**. Interestingly, although DNA-PK activity cannot be up-regulated by strong doses of radiation, we found that NO can act a signal, increasing the activity of DNA-PK. Importantly, we showed that this increase occurred by transcriptional up-regulation of DNA-PKcs expression and occurred under physiologically relevant ranges of NO concentrations **(Xu *et al*., 2000).** Biologically, this NO-mediated increase in enzymatically active DNA-PK not only protected cells from the toxic effects of NO, but also provided cross-protection against clinically important DNA-damaging agents, such as X-ray radiation, adriamycin, bleomycin and cisplatin **(Xu *et al*., 2000).**

The NO-mediated increase in DNA-PKcs pathway not only plays an important role in tumour DNA repair (**Kolb, 2001)**, but may also play an important role in other tissue damage processes which involve NO-mediated stress (**Bartunek *et al*., 2002).** For example, failing myocardium (advanced heart failure due to idiopathic dilated cardiomyopathy) undergoes active DNA repair, where DNA-PKcs expression is strongly correlated with iNOS expression **(Bartunek**

***et al*., 2002).** Given the fact that one of the major substrates of DNA-PKcs is p53 (**Woo *et al*., 1998).**

NO-based therapeutics can be traced back for more than a centrury when Willaim Murrell proposed the sublingual application of nitroglycerin as a remedy for angina pectoris (**Murrell, 1879).** From the time of discovery of the vasodilatory properties of the organic nitrates and nitrites, it took more than hundred years to elucidate their mode of action at the molecular level. For example, it was not until 1987 that NO gas was identified both as the endogenous endothelium-derived relaxing factor and as being involved as a primary defense mechanism against tumour cells and intracellular microorganisms (**Hibbs *et al*., 1987).**

Several laboratories have demonstrated that NO-releasing agents can kill tumour cells, and consequently there have been attempts to deliver NO to cells. While NO-releasing drugs are under development, an attractive alternative mechanism for delivery would be to transfer NOS encoding cDNA sequences into cancer cells for gene therapy purposes. Several studies have shown that this approach may work. For example, using a mouse model it was demonstrated that transfection of K-1735 melanoma cells with an iNOS cDNA expression cassette suppressed tumourogenicity and abrogated metastasis (**Xie *et al*., 1995).** Transfection of human renal carcinoma cells with a retroviral iNOS cassette showed similar results (**Juang *et al*., 1998).** A problem with current approaches, however, is that constitutive expression of NOS can quickly result in death of the transfectant, shortening the time that NO can be generated, and potentially limiting the utility of the approach. NOS transfectants often have to be cultured under conditions that reduce toxicity (for example in the presence of a NOS inhibitor), and transfection attempts may result in cells that are capable of relatively low levels of NO-generation (**Ambs *et al*., 1998).** As discussed above, this may result in concentrations of NO that promote tumour growth rather than cell killing (**Tzeng *et al*., 1996).**

Another significant point is that NOS enzyme activity requires a panel of substrates and cofactors for full activity, and these may be missing from the target cell type. For example, synthesis of the important cofactor tetrahydrobiopterin (BH4), requires transcriptional regulation of the rate limiting enzyme GTP-Cyclohydrolase, which may not be induced in all target cells (**Tzeng *et al*., 1996).** Lastly, both retroviral and adenoviral vector may be hazardous to the host and pose a major health and safety risk (**Lehrman, 1999).**

A potential strategy to overcome the problems associated with gene therapy is to use a cell-based approach. Cell-based approaches utilize the delivery of recombinant cells (rather than genes) to the target site, with the advantage that the expression of the gene of interest can be optimized

prior to delivery. For example, we have recently shown the utility of two novel iNOS-expressing human cell lines that can generate high concentrations of NO following treatment with analogues of either the insect hormone ecdysone or tetracycline (**Xu *et al.*, 2002)**. In order to make the NO-generating cells suitable for therapeutic delivery they have been encapsulated within a semipermeable alginate-poly-L-lysine membrane. Encapsulated cells are protected from environmental stresses encountered in the host (such as the host immune response) and can be delivered to the tumour site(s) in a nude mouse model (**Xu *et al.*, 2002).** Following delivery, high concentrations of NO and reactive nitrogen species can be generated by administration of the appropriate inducer. This approach has been very successful, and we have used it in a tumour model showing 100% killing of SKOV-3 tumours and 54% killing of DLD-1 tumours (**Xu *et al.*, 2002)**. Importantly, this strategy allowed the mechanism of tumour killing to be determined as it was shown that tumour killing was associated with concomitant up-regulation of the Fas/FasL proteins. Overall we believe that the cell-delivery approach addresses some of the shortcomings of competing strategies and has the potential to inhibit or kill many different types of tumours from various histologic origins (**Hahne *et al.*, 1996).**

7. 3. NO and obesity:

Nitric oxide (NO) is a free radical gas that acts as apleiotropic transmitter in many diverse functions (**Bredt, 1999).** Most importantly, its production by endothelial NO synthase (eNOS) mediates vasodilation and inhibits thrombocyteaggregation. In other cells such as immune cells or neurons, NO is produced by the inducible NO synthase (iNOS) and neuronal NO synthase (nNOS), and mediates an inflammatory response or acts as an atypical neurotransmitter, respectively **(Morley and Flood, 1994).**

Furthermore, it was suggested that NO plays an important role in the regulation of energy balance because administration of the non-specific NO synthase inhibitorN-nitro-l-arginine-methyl ester (L-NAME) reduced weight gain and food intake in mice **(Morley and Flood, 1994).** In nNOS-knockout mice, the appetite-suppressant activity of leptin was markedly reduced, suggesting that NO is a downstream signal of leptin (**Calapai *et al.*, 1999).** Because deletion of the iNOSprotected mice against high-fat diet-associated insulin resistance (**Perreault and Marette., 2001).** It was also suggested that NO "nitrosativestress" is a player in the pathogenesis of insulin resistance (**Kaneki *et al.*, 2007).** However, such a link between NO and insulin sensitivity is not consistent with studies in eNOS knockoutmice, in which deletion of one allele led to reduced insulin sensitivity in response to a high-fat diet (**Cook *et al.*, 2004),** and with theconcept that endothelial dysfunction is reciprocal to in-sulin sensitivity (**Kim *et al.*, 2006).**

In this issue of Endocrinology, he deals with the role of NO in regulating energy balance, supports the view that NO synthase is involved in this process, and offers potential mechanical links with obesity and diabetes (**Barish *et al*., 2006).**

The authors demonstrate that asynchronous application of an installed NO synthase inhibitor L-NAME significantly reduced body weight in mice. Intriguingly, this effect was larger in mice on a high-fat diet; here, L-NAME markedly reduced the volume of fat cells as well as triglyceride accumulation. Consistent with this phenomenon, L-NAME reversed the detrimental effects of the high-fat diet on hepatic triglyceride content, glucose tolerance, and in vivo insulin sensitivity. The authors conclude that these effects are due to increased energy dissipation because L-NAME elevated the mRNA levels of uncoupling proteins 1 and 3 in muscle and brown adipose tissue, respectively. Most interestingly, L- NAME also increased the expression of peroxisome proliferator-activated receptor in muscle. This transcription factor has recently been shown to be of central importance for fat oxidation in muscle (**Barish *et al*., 2006).**

In addition, generation of NO in mitochondria by the mitochondrial NO synthase reduces respiration and oxygen consumption (**Giulivi *et al*., 2006).** The inhibition of NO synthesis partially protects mice from the adipogenic effects of a high-fat diet. However, the study leaves a few questions unanswered. The most important, the here presented data do not provide any direct evidence for an increase in energy expenditure or an enhanced oxidation of fatty acids in muscle in response to L-NAME. Furthermore, there is a striking difference between the present results and those by Morley and Flood (**Morley and Flood, 1994).** In the previous study, administration of L-NAME for 9 days produced a significant inhibition of food intake in obese mice. In contrast, reduced adiposity observed in the present study could not be explained by reduced caloric intake. However, it should be noted that small changes of daily food intake or energy expenditure may lead to large effects on adiposity over time. Morbidly obese mice such as the New Zealand obese mouse consume approximately10% more calories and have a slightly lower body temperature (by 0.7 C) than lean controls (**Jugens *et al*., 2006**). Quantification of even smaller differences is technically challenging. Finally, it has to be assumed that the effect of L-NAME represents the net result of a non-specific inhibition of eNOS, iNOS, and other isotopes. According to the previous data, these tissue-specific isotopes appear to exert diverging effects on insulin sensitivity (**Cook *et al*., 2004).**

Unfortunately, global inhibition of NO synthesis will lead to undesired cardiovascular and other side effects. L-NAME produced a substantial increase of blood pressure, from 112–144 mm Hg, and a compensatory reduction in heart rate. Such side effects would certainly represent a

difficult obstacle for a chronic drug therapy of obesity. Thus, without dissociation of vascular effects of those on energy balance, possibly by a selective and tissue-specific inhibition of iNOS in muscle and/or adipose tissue, it is hard to envision a therapeutic benefit of this approach. After the discovery of leptin obesity research has been one of the fastest-moving fields, and has provided a large body of knowledge as to the neuroendocrine and hormonal control of hunger, satiety, and energy expenditure **(Coll *et al*., 2007).** Furthermore, a substantial number of genes involved in the regulation of fat storage has been identified through their knockout phenotypes. In contrast there is much less progress in the search for new therapeutics of obesity, and very few "druggable" targets have been identified so far. The few candidates that made it through large clinical trials, such as cannabinoid receptor antagonists, struggle due to their limited efficacy, potential side effects, and small therapeutic window **(Tsuchiya *et al*., 2007).**

A large number of unknown or untested obesity targets exist, awaiting detailed examination. Among the genome-wide mutations or knockout screens in Drosophila melanogaster, the total number of genes involved in regulating energy balance can be estimated in the hundreds **(Dohrmann, 2004)**. Data from mice are consistent with this estimate. A recent meta analysis of genome-wide searches for susceptibility loci (quantitative trait loci) indicated that at least 50 chromosomal segments containing alleles associated with obesity or thinness have been identified **(Wuschke *et al*., 2007).** Identification of these by positional cloning is difficult and time consuming but appears feasible based on rapidly evolving technology **(Buchmann *et al*., 2007)**. Furthermore, genome-wide association studies in humans carry the potential to lead to new insights into the mechanisms of triglyceride storage; proof of principle has been provided by the recent discovery of the fat mass and obesity associated (FTO) gene **(Frayling *et al*., 2007).**

7.4. Nitric oxide and gastrointestinal disease:

Evidence indicates that NO may be an endogenous vasodilator regulating gastric mucosa blood flow and maintaining mucosal integrity and defense. In the rat stomach, inhibition of NO synthesis reduces gastricmucosal blood flow as measured by a hydrogen gas clearing technique, indicating that endogenous NO modulates basal tone in the gastric vasculature **(Pique *et al*., 1989)** Increases in gastric mucosa blood flow induced by acetylcholine or bradykinin in the rat is endothelium-dependent, and NO synthase inhibitors attenuate pentagastrin-induced increases in gastric mucosa blood flow **(Pique *et al*., 1992).**

Endogenous NO may contribute to mechanisms that protect against ulcerations of the gastric mucosa. Topicalmucosal application of a NO solution or of glyceryl trinitrate or nitroprusside **(Feelisch *et al*., 1987).** Which release NO Reduces the severity of ethanol-induced

hemorrhagicmucosal damage **(MacNaughton *et al*., 1989).** Intravenous administration of nitroprusside also inhibits mucosal damage. Administration of inhibitors of NO synthesis alone did not lead to acute gastric mucosa damage in the rat. However, inhibition of NO synthesis after depletion of vasodilator neuropeptides with chronic apsaicin pretreatment or after inhibition of post acyclin synthesis with indomethacin did induce substantial mucosal injury **(Whittle *et al*., 1990)**. Another study suggests that the gastroprotective effect of sensory neuropeptides released by acute capsaicin exposure is mediated by NO. These results suggest a role for endogenous NO, interacting with prostacyclin and vasodilator neuropeptides in the regulation of gastric mucosa integrity **(Peskar *et al*., 1991).**

The mechanism underlying the mucosal protective actions of NO is not known. It seems likely that vasodilation or inhibition of platelet aggregation in the gastric microvasculature are involved, but it impossible that NO has other actions in the gastric mucosa that enhance or preserve epithelial cell function and continuity. The source of the gastro-protective endogenous NO is also not known. The vascular endothelium seems a likely source, but given the recent demonstrations of NO formation in many cell types, other sources including white blood cells, epithelial cells, or neurons should also be considered **(Ignarro, 1990).**

7.5. Role of Nitric oxide in lung disease:

Endogenously produced nitric oxide plays a major role in lung physiology and pathology. Inhaled nitric oxide given exogenously has been studied extensively as a treatment for many lung diseases, and the results suggest that it may help improve oxygenation in some patients (**Raed, 2001).**

NO plays a well-established role in the endothelial-dependent control of vascular tone and in mediating vascular smooth muscle relaxation in the pulmonary circulation. Nitric oxide is a potent vasodilator in the bronchial circulation and may play an important role in regulating airway blood flow. It also modulates vascular tone through its vasodilatory properties. Excess amounts of NO may cause the hypotension that is associated with sepsis. Decreased NO levels within the lungs may contribute to the pathological states associated with pulmonary hypertension airway effects**.** NO promotes bronchodilation by directly relaxing the smooth muscles in the airway. Produced continuously by the overlying airway epithelium, NO can diffuse easily into the bronchial smooth muscle and cause smooth muscle relaxation through the activation of

guanylylcyclase to produce guanosine cyclic monophosphate. In addition, NO can directly affect bronchial tone because it is the neurotransmitter of the inhibitory non-adrenergic, non-cholinergic broncho-dilator nerves. Nitric oxide generated by constitutive NOS in these nerves is thought to have a broncho dilatory effect. NO may also play a critical role in ventilation-perfusion coupling in the lung. This theory is supported by the fact that endogenous NO levels in the lung change rapidly in direct proportion to inspired oxygen (**Scherrer *et al*., 1996).**

7.5.1. Asthma:

Patients with asthma have high levels of NO in their exhaled breath and high levels of NOS II enzyme expression in the epithelial cells of their airways. Both exhaled NO and NO II expressions return to normal levels after treatment with corticosteroids (**Dweik*et al*., 2001).**

Although these findings suggest that, NO plays a role in asthma pathogenesis, its exact role in airway reactivity remains elusive. Because NO is a bronchodilator, it is possible that the high levels of NO have a beneficial effect on the airway. On the other hand, NO could be involved in the pathogenesis of asthma by modifying bronchial hype responsiveness or the underlying inflammation, or it may be a simple marker of inflammation. Furthermore, because NO has both inflammatory and anti-inflammatory properties, it can modulate the underlying inflammation in asthma. Thus, the explanation for the elevated levels of NO in asthma has turned out to be more difficult than initially thought. (**Dweik*et al*, 2001).**

7.5.2. Primary pulmonary hypertension:

Patients with primary pulmonary hypertension have decreased levels of NO in their lungs, which may contribute to the development of pulmonary hypertension. Besides being a potent vasodilator, NO also inhibits proliferation of vascular smooth muscle and alters gene expression of several growth factors (e.g., endothelial growth factor and platelet-derived growth factor). An NO deficiency may facilitate the proliferation of vascular cells and remodeling of the pulmonary vasculature. Recently, they performed fiber-optic bronchoscopy in patients with primary pulmonary hypertension to measure intrabronchial NO and NO biochemical reaction products (**Kaneko *et al*., 1998)**. They found that NO and the reaction products of NO are reduced in patients with primary pulmonary hypertension compared with healthy individuals. Interestingly, the low levels of NO products correlated directly with the degree of pulmonary arterial hypertension. This evidence indicates that levels of vasodilators are directly related to constriction of the pulmonary

vasculature and likely contribute to the pathogenesis of primary pulmonary hypertension (**Kaneko *et al*., 1998)**.

7.6. Nitric oxide and Neurological disease:

7.6.1. Parkinson's disease:

Parkinson's disease (PD) is a neurological disorder characterized by degeneration of dopaminergic neurons in the substantia nigra Zona compacta. The cause of sporadic PD is still unknown although increasing evidence indicates that it may represent the outcome of a complex set of interactions, over decades of time, among factors that include genetic predisposition, innate characteristics of the nigrostriatal dopaminergic system of the brain, and exposure to environmental toxins **(Kidd, 2000).**

In recent years, an involvement of altered activity of NO synthases (NOSs) (mainly the neuronal and inducible forms) has emerged. In fact, high levels of neuronal NOS (nNOS) and inducible NOS (iNOS) expression were observed in the nigrostriatalregion and basal ganglia in the post-mortem PD brains. NO has been also proposed to have a role in the inflammatory processes occurring in PD (**Whitton, 2007).**

The role of NO in the pathogenesis of PD is still controversial. Some research groups reported data showing no correlation between PD and NO **(Shukla *et al*., 2006).** On the contrary, a large number of groups suggest that NO could be regarded as one of the factors contributing to oxidative stress and oxidative damage evidenced in post-mortem studies, in vitro and animal experimental models **(Kavya *et al.*, 2006).**

PD is the most common age-related neurodegenerative disease after Alzheimer's disease and is grouped among motor system disorders. PD is a slow and progressive disease, mainly characterized by resting tremor, slowness of movement (bradykinesia), stiffness (rigidity), and poor balance (postural instability). Beside mitochondrial dysfunctions, inflammation, apoptosis, oxidative and nitrosative stress, mutated genes were also found in familial PD these include genes encoding for mitochondrial proteins such as Parkin, PTEN-induced putative kinase 1 (PINK1), DJ-1, mitochondrial polymerase gamma 1 (POLG1), and non-mitochondrial proteins such as a-synuclein, leucine-rich repeat kinase 2 (LRRK2). A significant number of researchers found an association between the expression of such mutated proteins and the occurrence of redox status and/or mitochondrial homeostasis alteration in PD models, thus reinforcing the relevance of

oxidative stress and mitochondrial dysfunction in the pathogenesis of familial and sporadic forms of the disease (**Schapira, 2006).**

The implication of NO in PD has been firstly proposed when high levels of nNOS and iNOS were found in the nigrostriatal region and basal ganglia of the post mortemPD brains (**Eve *et al.*, 1998)**. Immunoreactivity for nNOS and downstream effector guanylate cyclase were also found consistently increased in the substantia nigra after treatment with PD-inducing drugs **(Chalimoniuk *et al.*, 2004).** It has been suggested that a significant portion of the increased NO production derives from the induction of iNOS as consequence of the inflammatory processes due to microglia activation **(Whitton, 2007).**

The interaction of several pathogenic pathways concurs to PDs how that NO represent an important downstream mediator and enhancer of molecular events leading to dopaminergic neurons death. As below reviewed, NO overproduction appears to be an event that significantly contributes to death of dopaminergic neurons via oxidative damage on cellular lipids, proteins and DNA. This hypothesis is supported by studies reporting that NOSs inhibitors or NOSs gene knockout significantly mitigate nigral cell loss in animalexperimental models **(Cutillas *et al.*, 1999).**

7.6.2. Alzheimer's disease:

Alzheimer's disease (AD) is the most common cause of dementia in older individuals. AD is a neurodegenerative disorder characterized by a progressive and global deterioration in mental function, most notably in cognitive performance. Progressive impairment of memory and visual and spatial cognition are accompanied by changes in affective behavior, including depression and aggression, leading to disintegration of intellectual skills, personality, and the ability to function in everyday life **(Bernabeu *et al.*, 1997).**

Recent studies show that NO/sGC/cGMP signaling is important in multiple forms of synaptic plasticity, and several reports have provided experimental evidence suggesting that the sGC/cGMP signal transduction system is important for acquisition of new learning and memory. Passive avoidance learning in the rat is associated with an increase in the level of cGMP in the hippocampus, and administration of the membrane permeant cGMP analogue, 8-bromo cGMP, enhances memory performance. Conversely, in the same paradigm, inhibition of either sGC activity or cGMP dependent protein kinase (PKG) immediately post-training blocks memory formation **(Bernabeu *et al.*, 1997).** Selective inhibition of Nnos with 7-nitroindazole impairs object recognition memory in rats, whereas treatment with zaprinast, a selective cGMP phosphor

diesters inhibitor, both facilitates object recognition and reverses the memory deficit induced by 7-nitroindazole **(Prickaerts *et al*., 1997).** Post-training infusion of 8-bromo-cGMP bilaterally into the hippocampus improves object recognition memory, whereas 8-bromo-cAMP is ineffective **(Prickaerts *et al.*, 2002).**

The animal studies that implicate the NO/sGC/cGMP signal transduction system in learning and memory are supported by numerous *in vitro* studies showing that long-term potentiation (LTP) in the hippocampus can be blocked by inhibition of sGC **(Bon *et al*., 2003).** NO and cGMP can induce long-lasting enhancement of presynaptic neurotransmitter release **(Kendrick *et al*., 1997).**

Furthermore, the close temporal relationship between activation of the NO/sGC/cGMP signal transduction cascade and improvements in learning and memory suggest a mechanistic link between the two phenomena **(Bon *et al*., 2003).** Activation of sGC leading to cGMP accumulation will activate PKG that in turn initiates protein phosphorylation cascades leading to activation of transcription regulating factors such as cAMP response element-binding protein (CREB), a critical event in both LTP and the establishment of long-term memory **(Chien *et al.*, 2003**). Acetylcholine plays a critical role in modulating synaptic function in the cerebral cortex and hippocampus. The precognitive actions of ACh in these brain regions are mediated *via* activation of muscarinic receptors, which induce primarily excitatory effects involving multiple different ionic conductances **(Krause *et al*., 2000).** In the hippocampus, cholinergic muscarinic receptor activation leads to increased tissue levels of cGMP (**Sirvio, 1999).**

Importantly, the inhibition of the slow after hyperpolarizing current (a calcium-activated potassium conductance that underlies spike-frequency adaptation) induced by muscarinic receptor activation in hippocampal CA1 pyramidal neurons can be blocked by inhibitors of sGC andPKG **(Krause *et al*., 2000).** Therefore, the neuro-modulatory effects of ACh in the brain must, at least in part, involve NO/sGC/cGMP signaling. Behavior al studies have demonstrated that NMDA receptors also play an important role in both spatial working memory and long-term memory processes. Blockade of NMDA receptors, or inhibition of NOS activity, impairs performance in the Y-maze test, a model of spatial working memory **(Yamada *et al*. 1996).** The impairment induced by NMDA receptor blockade could be reversed by intracerebroventricular administration of the nitrosothiol *S*-nitroso-*N*-acetylpenicillamine (which acts in part as NO donor), L-arginine (the substrate for NOS), or dibutyrl-cGMP **(Yamada *et al.*, 1996).**

From these studies it can be proposed that both ACh and glutamate activate receptor systems coupled to NO/sGC/cGMP signal transduction and that this biochemical pathway is

important for synaptic plasticity and the formation of memory. NO-stimulated sGC activity is severely decreased in the cerebral cortex of patients with AD, and aberrant signaling by NO has been reported to occur in the brain in AD **(Lu *et al.*, 1999)** These findings lead to the prediction that sGC activation and cGMP formation in the brain may be an effective strategy for mitigating the cognitive dysfunction that occurs as a consequence of cholinergic deficits in the CNS. Therefore, in contrast to the cholinesterase inhibitors that attempt to salvage the functionality of a degenerating cholinergic system, NO mimetics are postulated to bypass this system, to modulate the normal function of signaling pathways downstream from cholinergic receptor activation **(Lu *et al.*, 1999).**

7.7. Nitric oxide and cardiovascular disease:

The reduced bioavailability of NO is thought to be one of the central factors common to vascular disease, although it is unclear whether this is a cause of, or result of endothelial dysfunction. There are a number of factors which could potentially affect either the production of NO, or the ability of NO to diffuse to its cellular targets. Disturbances in NO bioavailability lead to altered regulation of key physiological and cellular processes such as vasodilatation, platelet function, angiogenesis, apoptosis and smooth muscle cell proliferation. In the following section, the factors affecting both the production and availability of NO will be discussed. Furthermore these changes in NO bioavailability will be discussed in the contextof the physiological processes they regulate. It is important to highlight these factors and explore their importance both individually and in combination **(Beckman *et al.*,1994).**

The increased production of O2 could be critical to NO bioavailability and disease progression, due to the possible formation of peroxynitrite. Seminal work by Beckman et al. (1994) demonstrated a high degree of staining for nitrotyrosine in atherosclerotic plaques at post-mortem, suggesting that increased levels of peroxynitrite are formed in the areas of atherosclerosis **(Beckman *et al.*,1994).** The effect of peroxynitrite formation in areas of atherosclerosis is two-fold firstly, loss of bioavailable NO, and secondly, the formation of secondary and tertiary pro-atherosclerotic oxidants. It should be made clear that the main source of the NO which promotes oxidation of lipoproteins in the form of peroxynitrite is likely to be the macrophages rather than the endothelium. Nevertheless, the loss of NO would abrogate its anti-platelet and anti-leukocyte actions. However, the pro-oxidant effects of peroxynitrite formation are for more damaging. Peroxynitrite, but not NO, has the capacity to induce the oxidation of LDL, a critical event in the development of atherosclerotic plaques (**Graham *et al.*, 1993).**

Unlike other reactive species, peroxynitrite has the capacity to modify both lipid and protein moieties of LDL. Peroxynitrite can deplete LDL of the antioxidants, a-tocopherol and b-carotene, and induce the oxidation of lipids to produce lipid hydroperoxides and isoprostanes **(Patel *et al.*, 2000)**. Protein moiety of LDL, apoB100, is modified by both direct and indirect mechanisms. Peroxynitrite directly oxidizes cysteine and tryptophan, while modification of lysine and arginine probably occurs via secondary reactions with lipid hydroperoxide radicals. The modification of LDL converts it to a highly atherogenic particle, which promotes SMC dysfunction and foam cell formation. There is strong evidence for this as the fingerprint of peroxynitrite, the presence of nitrated proteins is present in atherosclerotic plaque as originally shown by Beckman et al. (1994) and the location of these modified proteins is close to that of the macrophages in the plaque. Interestingly peroxynitritealso causes the oxidation of BH4 rendering it inactive (**Kuzkaya *et al.*, 2003**). It has been suggested that the beneficial effects of BH4 supplementation in atherosclerosis maybe by replacing lost BH4.The excess production of NO by macrophages may have another negative effect. Asindicated earlier NO keeps smooth muscle cells in their contractile phenotype and prevents proliferation to the fibro blastic form. In the reparative phase, excess NO may impair the formation of the fibrous cap or reduce the mechanical strength of the cap. Macrophage accumulation appears to reduce the thickness of this cap. However, surgically induced atherosclerosis in animal models, which is mainly due to smooth muscle cell proliferation after vein grafting, increased availability of NO by gene transfection of NOSII prevents this occurring and keeps the new vessels open **(Schwentker and Billiar., 2002**).

7.7.1. Nitric oxide and hypertension:

NO is crucial to the maintenance of normal blood pressure **(Huang *et al.*, 1995)** and therefore its relationship to essential hypertension has been the subject of intense investigation. As with coronary artery, disease and diabetes initial evidence suggesting NO-dependent component of the disease came from studies assessing endothelium-dependent vasodilatation. A number of studies have demonstrated the impairment of NO-mediated vasodilatation in brachial (**Panza *et al.*, 1995),** coronary and renal arteries (**Higashi *et al.*, 1995**) in patients with essential hypertension compared to controls **(Kelm, 2003**).

The reason for reduced NO dependent vasoactivity is unclear. Evidence suggests that the impaired NO responses are genetically determined, since basal NO production is impaired in offspring of patients with essential hypertension **(Kelm. 2003**). This work also suggests that impaired NO production does not occur simply because of the disease but may be integral to disease development. The possible genetic component of the disease has led to a plethora of

studies assessing the role of NOS polymorphisms in essential hypertension. As with many other studies, the results are inconclusive **(Miyamoto *et al*., 1998).**

The polymorphism (894G to T) in exon 7 causes the conversion of Glu to Asp at position 298 and is thought of as one of the most promising polymorphisms in relation to disease. Thispolymorphism has an increased frequency in hypertensive compared to controls and was associated with a resistance to antihypertensive therapy, although NOS activity was not addressed **(Miyamoto *et al.*, 1998).** Similarly, a multi-centred assessment of the polymorphism in Japan found an increased frequency in the hypertensives **(Shoji *et al.*, 2000).** However, a study assessing as similar number of hypertensive subjects found no difference in polymorphisms frequency **(Lacolley *et al*., 1998).** Other studies have addressed the frequency of a number of different polymorphisms in hypertension with varying degrees of success **(Wang and Wang. 2000).**

Essential hypertension is a multifactorial disease where the interactions between neuronal, hormonal and cellular signaling processes all contribute to the pathogenesis. eNOS gene polymorphisms probably led to only very subtle changes in NO production, and thus the contribution of these polymorphisms may only become important in presence of other contributing factors. Therefore, the group of patients used for each study is a critical determinant of the results. Several studies have demonstrated impaired endothelial dysfunction in essential hypertension, which is associated with a blunted response to NO-mediated effects. Although it is unclear whether this represents reduced synthesis or increased consumption of NO. The vasodilator effects of the NO-donor sodium nitroprusside were identical in both hypertensive and normotensives **(Calver *et al.*, 1992),** suggesting that the cGMP-signaling pathway in smooth muscle cells is normal. Interestingly, hypertensive subjects have defective vascular responses to acetylcholine, but normal responses to b-adrenergic stimulation by bradykinin. These findings suggested that endothelial dysfunction associated with essential hypertension is due to a selective abnormality of the signaling pathways leading to NOS activation. Indeed, the hypotensive effects of angiotensin-converting enzyme (ACE) inhibitors in hypertensive subjects, has been shown to occur through increased formation of bradykinin and leading to enhanced NO formation (Ignjatovic et al., in press) (**Achan *et al.*, 2003)**.

Another study has suggested that the depressed levels of basal NO in hypertensive subjects could be due to increased circulating levels of ADMA (**Achan *et al.*, 2003),** although this has not been confirmed. The spontaneously hypertensive rat model was also used to show that NO production was normal, but O-2 production is elevated and led to increased oxidation of NO and resulting in decreased vasodilatation **(Heitzer *et al*., 1999**). The O2 could arise from a variety of

different sources including NAD (P) H oxidases and cyclo-oxygenase activity, since the activity of both enzymes is seen to be unregulated in hypertension **(Heitzer *et al*., 1999**).

This evidence is corroborated in human subjects where infusion of ascorbic acid reversed the impaired endothelium-dependent relaxation in hypertensive, again suggestive of an important role for oxygen free radicals (**Taddei *et al*., 1998).**

7.7.2. Role of NO in atherosclerotic disease:

Atherosclerosis is the result of hyperlipidemia and lipid oxidation and has always been a major cause of mortality in developed countries. It is a disease of vascular intima, in which all the vascular system from aorta to coronary arteries can be involved and is characterized by intimal plaques **(Hennekens and Gaziano., 1993).**

The term atherosclerosis is of Greek origin, meaning thickening of the intimal layer of arteries and accumulation of fat. Fatty material is located in the central core of the plaque, covered by fibrous cap. The term, atherosclerosis consists of two parts; athetosis (accumulation of fat accompanied by several macrophages) and sclerosis (fibrosis layer comprising smooth muscle cells [SMC], leukocytes, and connective tissue) (**Ross, 1999).**

7.7.2.1. Nitric oxide and Cholesterol:

Nitric oxide (NO), the endothelium-derived relaxing factor. Originating from metabolism of L-arginine can prevent atherosclerosis by inhibiting oxidation of lipoproteins in the arterial wall **(Jessup., 1996).** In addition, some studies have suggested that NO can modulate metabolism **(khedara *et al*., 1996)** of lipoproteins when its availability is altered by administration of synthetic NO synthase inhibitors or NO donors. In cholesterol-fed rabbits, chronic administration of the NO synthase inhibitor, N -nitro-L-arginine. L-NAME promoted atherosclerosis and tended to increase hypercholesterolemia **(Naruse *et al*., 1994).** Similarly, serum cholesterol level was moderately elevated in rats treated with another NO synthase inhibitor, L-N Nitroarginine. L-NNA (**Khedara *et al*., 1996).** Conversely, a substantial, dose-dependent reduction of hypercholesterolemia as well as atherosclerosis was observed in cholesterol-fed Japanese quail after chronic oral administration of a NO donor, sodium nitroprusside NaNP **(Hill *et al*., 1995**).

A relationship between activity of NO and cholesterol has been found in the absence of pharmacological intervention. In rabbits with diet-induced hypercholesterolemia, urinary excretion of NO metabolic products, nitrites, was decreased **(Bo¨ger *et al*., 1995)** and plasma levels of an endogenous NO synthase inhibitor, NG, NG-dimethylarginine, were increased,

suggesting impaired NO activity **(Bode *et al*., 1996)**. In agreement, an excessive release of NO during inflammation and tissue injury has been associated with acquired hypercholesterolemia, although this response has been postulated to be at least partly due to preceding release of cytokines **(Ettinger *et al*., 1994).**

Previous results suggested that NO-mediated hypercholesterolemia responses are unlikely to be enhanced by increased dietary intake of NO precursor, L-arginine. Dietary supplementation with L-arginine has been shown to reverse endothelial dysfunction caused by hypercholesterolemia and to inhibit atherogenesis **(Cooke and Tsao, 1997)** but failed to counteract hypercholesterolemia it self (**Aji *et al*., 1997)**, dietary L-arginine appeared to have anti- hypercholesterolemic properties in rabbits fed hypercholesterolemic amino acid diets as well as apo B-lowering properties in HepG2 cells (**Kurowska and Carroll, 1996).** However, in the rabbit model, feeding high levels of L-arginine was not associated with increased plasma and liver content of NO and metabolites, nitrates unpublished data. Another possibility that a stable intermediate precursor in L-arginine-NO pathG way, N -hydroxy-L-arginine N–OH–Arg, could be important in NO-mediated regulation of lipoprotein metabolism is yet to be investigated. It has been shown that in vitro, N–OH–Arg can be oxidized to NO and nitrite in the absence of active NO synthase, probably by cytochrome P **(Schott*et al*., 1994).**

7.7.2.2. Nitric oxide and triglycerides:

Hypertriglyceridemia is a prevalent risk factor for cardiovascular disease (CVD) and increasingly important in the setting of current obesity and insulin resistance epidemics. High triglyceride (TG) levels are markers for several types of atherogenic lipoproteins. Patients who have hypertriglyceridemia may be at significant risk for CVD even if low-density lipoprotein cholesterol levels are at goal, and therefore warrant treatment that optimizes diet, reduces overweight, and promotes regular exercise **(Beatriz and Frank., 2011).**

Nitric oxide play roles in blood vessel dilation, immune reactions, and the central and peripheral nervous systems **(khedara *et al*., 1999**). NO production is enhanced by estrogen, inflammation, and exercise through elevation of NO synthase activity. NO is inactivated by reaction with superoxideanion, and oxidative stress causes lower level of NO, which in turn causes some aggravation effects such as hypertension. (**Goto *et al*., 1999**) Researchers have found that feeding L-N ^{w} nitroarginine (L-NNA), a specific strong inhibitor of NO synthase, to rats caused higher concentrations of serum triglyceride, A diet containing elevated L-NNA serum nitrate by competitive inhibition of stress NO synthase by L-NNA, and suppression of elevations of these fats in serum. **(Khedara *et al*., 1996),** where nitric oxide production is closely related to lipid

concentrations in the blood **(Jong, 2004**). This indicates that nitric oxide it may play an important role in regulating fats in the body **(khedara *et al*., 1999**).

7.7.2.3. Nitric oxide and lipoproteins:

The mechanism by which changes in activity of NO can influence the metabolism of lipoproteins is poorly understood. The lipoprotein responses produced in animals treated with inhibitors of NO synthase, although obscured by cholesterol feeding, suggested that VLDL and/or LDL rather than HDL cholesterol are likely to be affected **(Naruse *et al*., 1994).** Since VLDL and LDL are synthesized and catabolised in the liver, they hypothesized that regulation of lipoprotein metabolism by NO, directly or via its intermediate precursors, L-arginine or N–OH–Arg, could occur in this organ. To understand the nature of this regulation better, they investigated effects of exogenous NO donors on metabolism of lipoproteins in vivo and in vitro. In vivo, effects of 0.03% NaNP supplementation on lipoprotein profile and on liver lipids was tested using rabbits in which experimental hypercholesterolemia associated with elevation of LDL cholesterol, similar to that in humans, was induced by feeding a semi purified, cholesterol-free, case in diet **(Hrabek-Smith *et al*., 1989)**. In vitro, cholesterolemic responses were analyzed in human hepatoma HepG2 cells after their exposure to a less toxic NO donor, S-nitroso-Nacetylpenicillamine SNAP **(Hrabek-Smith *et al*., 1989)**.

Experiments were also conducted to determine whether in HepG2 cells, the apo B-lowering effect induced by incubation with high levels of L-arginine could be mediated by NO and whether a similar reduction of apo B in the medium could be obtained by exposure of HepG2 cells to N-OH-Arg. **(Hrabek-Smith *et al*., 1989).**

The hypertriglyceridemia by L-NNA was mainly attributable to a higher concentration of triglycerides in the VLDL fraction. As the concentrations of cholesterol and phospholipids in the VLDL fraction were elevated by L-NNA, L-NNA treatment caused elevated serum-free fatty acids and decreased hepatic CPT activity (an enzyme that limits the rate of fatty acid oxidation) without affecting hepatic activities. As well as higher PAP and lower CTP activity in microorganisms by L-NNA. Hence, PAP and CTP are involved in the rate determination step of triglyceride synthesis and phosphatidylcholine synthesis, respectively, and appear to be present in both soluble and particle forms synthesis by increasing fatty acid esterification and liver low fatty acid oxidation, leading to elevations in serum and VLDL triglycerides. On the other hand, phosphatidylcholine synthesis may also indirectly enhance triglyceride synthesis by increasing the fatty acids available for triglyceride synthesis **(Goto*et al*., 1999).**

Conclusion and Perspectives

Conclusion and perspectives:

Nitric oxide (NO) is a ubiquitous free radical that is synthesized from an L-arginine molecule in a sequential oxidation reaction.

NO production is catalyzed by NO synthases (NOS). There are three distinct isoforms of NOS, endothelial form (eNOS) and neuronal (nNOS) are constitutively active and synthesize NO in response to increased intracellular Ca^{2+}. The third one is the inducible form (iNOS). The three isoforms play important roles as dichotomous effects in human biology and diseases, its effects depend on NO concentration and composition of intra-and extracellular milieu.

NO plays a very important role in the regulation of several physiological functions. There is an important association between serum/plasma NO levels and cardiovascular disease, hypercholesterolemia, heart failure, myocarditis, myocardial ischemia, atherosclerosis, hypertension, diabetes, thyroid disorders, metabolic syndrome and obesity. NO also has a strong trace in neurodegenerative diseases such as Parkinson's, amyotrophic lateral sclerosis, Alzheimer's disease and cerebellar degeneration.

The nitric oxide field has matured over the past 20 years to a point where both health care practitioners and patients are aware of NO and its effects on health and disease. Due to the ubiquitous nature of NO, technologies that can safely and effectively restore or recapitulate NO based signaling will have broad clinical utility.

Nitric oxide is known as an antimicrobial and anti-inflammatory molecule with major roles in pulmonary vasodilation, in the context of viral infections and other lung diseases. Therefore, further studies are needed to elucidate the other pathophysiologial roles of nitric oxide and its effets on several infectious diseases.

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ملخص

يتم الحصول على أكسيد النيتريك (NO) في مختلف الأنسجة بالتاثير الأنزيمي لـ NO synthase على الحمض اللأميني L-arginine. يتم تعزيز إنتاجه عن طريق الالتهابات،التمارين الرياضية وحمض الأسكوربيك وتثبيطه بالتفاعل مع فوق أكسيد الأنيونات.

يعتبرأكسيد النيتريك (NO) منظم خلوي مهم كما أن له أهمية في تنظيم العديد من الوظائف الفسيولوجية والعمليات المرضية. في نظام القلب والأوعية الدموية يقوم NO بتوسيع الأوعية الدموية وإرخاء العضلات الملساء الوعائية مما يسبب انخفاض ضغط الدم و يمنع تراكم الصفائح الدموية داخل الأوعية زيادة على وقف حدوث الجلطات الدموية. في الجهاز العصبي ، يعمل NO كناقل عصبي ويزيد من تدفق الدم والأكسجين إلى الدماغ. في الرئتين ، يوسع NO الأوعية الرئوية ويعتبرتركيزه في هواء الزفيرعلامة على التهاب مجرى الهواء. في الجهاز الكلوي يزيد تدفق الدم إلى الكلية. في الجهاز المناعي ينظم الاستجابة المناعية بوساطة الخلايا التائية.

في هده الدراسة تم تقديم بالتفصيل الأدلة والآليات الخلوية والكيميائية الحيوية التي تدعم مختلف وظائف أكسيد النيتريك.

الكلمات المفتاحية: أكسيد النيتريك ،Nitric oxide synthase ،L-Arginine.

Résumé

L'oxyde nitrique (NO) est formé par le clivage enzymatique de la L-arginine par la NO synthase dans plusieurs tissus. La production de NO est augmentée par l'inflammation, l'exercice, l'acide ascorbique et inactivée par la réaction avec l'anion superoxyde.

L'oxyde nitrique (NO) est un important régulateur cellulaire. Il a été démontré qu'il joue un rôle dans la régulation de tant de fonctions physiologiques et de processus physiopathologiques. Dans le système cardiovasculaire, le NO dilate les vaisseaux, détend les muscles lisses vasculaires provoquant une hypotension, inhibe l'agrégation des plaquettes dans les vaisseaux et prévient les événements thrombotiques. Dans le système nerveux, NO Agit comme un neurotransmetteur et augmente le flux sanguin cérébral et l'oxygénation vers le cerveau. Dans les poumons, le NO dilate les vaisseaux pulmonaires et sa concentration dans l'air expiré est un marqueur de l'inflammation des voies respiratoires. Dans le système rénal augmente le flux sanguin vers les reins. Dans le système immunitaire module la réponse immunitaire médiée par les lymphocytes T.

Les preuves soutenant ces rôles du NO et les mécanismes cellulaires et biochimiques menant à une biodisponibilité réduite du NO sont présentées en détail dans cette étude.

Mots clés: oxyde nitrique, oxyde nitrique synthase, L-Arginine.

Academicyear: 2019/2020 **Done by: MEGHRAOUI NAILA KHOULOUD and RIKOUAH MAISSA**

Dissertation to get Diploma of master in

Nutrition Biochemistry

Studies on the Different Role of Nitric Oxide

Abstract

Nitric oxide (NO) is formed by the enzymatic cleavage of L-arginine by NO synthase in several tissues. NO production is enhanced by inflammation, exercise, ascorbic acid and inactivated by the reaction with superoxide anion.

Nitric oxide (NO) is an important cellular regulator. It has been shown to play roles in the regulation of so many physiological functions and pathophysiological processes. In cardiovascular system, NO dilates vessels, relaxes vascular smooth muscles causing hypotension, inhibits the aggregation of platelets within the vessels and prevents thrombotic events. In nervous system NO Acts as a neurotransmitter and increases cerebral blood flow and oxygenation to the brain. In lungs NO dilates pulmonary vessels and its concentration in exhaled air is a marker of airway inflammation. In renal system, increases blood flow to the kidney. In immune system modulates T cell-mediated immune response.

Evidences supporting these roles of NO and the cellular and biochemical mechanisms leading to reduce NO bioavailability are presented in detail in this study.

Keywords: Nitric oxide, Nitric oxide synthase, L-Arginine.

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