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MINIREVIEW

Nitric Oxide: from a mysterious labile factor to the molecule of the Nobel Prize

Recent progress in nitric oxide research

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ABSTRACT

NO is now known to be an important messenger molecule in biology. It regulates a variety of functions within cells and tissues including vasodilation, neurotransmission and immunological process. This review will focus on the nitric oxide synthase gene family and recent progress on molecular genetic analysis of NOS1, NOS2 and NOS3 genes.

Key words: *Nitric oxide, NOS, gene family, genetic study.*

INTRODUCTION: Nitric Oxide and Nobel Prize

Scientific breakthrough often comes from unexpected observations, and imaginative adventurous research. In the early 80's at the New York Laboratory of Suny Health Centre, Dr. Robert Furchgott was presented contradicting results by his two technicians. One technician always found the acetycholine relaxed the blood vessel, whereas the other found it always caused contraction. Furchgott noticed that one technician handled the vessels roughly, and inadvertently rubbed off the thin layer of endothelium from the surface of the vessel, whilst the other was careful, and kept the endothelium intact. He then realized that an intact endothelium was a prerequisite for the relaxant effect of acetycholine[1]. By separating the endothelium from the smooth muscle, he showed that a labile factor was released from the endothelium,

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he named it the "endothelium-dependent relaxing factor" (EDRF).

Since then the race to hunt down this mysterious factor has been fiercely contested. The three winners of this race have been awarded the Nobel Prize for physiology/medicine this year. They are pharmacologists Robert Furchgott of the State University of New York; Louis Ignarro of the University of California, Los Angeles[2]; and Ferid Murad of University of Texas Medical School in Houston[3]. Although many scientists feel that a fourth name should also be recognized-Salvador Moncada, current director of the Wolfson Institute for Biomedical Research at University College London for his contribution to the conclusion that EDRF and nitric oxide was identical[4, 5].

NO regulates vasodilation, neurotransmission and immunological process

In late 80's these pioneer scientists discovered that a gas, Nitric Oxide(NO), previously considered to be merely atmospheric pollutant, is a critical signaling molecule for endothelium-dependent relaxation which occurs in response to a wide variety of stimuli, including acetylcholine, bradykinin, substance P, thrombin, adenine nucleotide and calcium ionophore A23187. It increases in the blood flow through arteries, microvessels and some veins. It is now clear that the endothelium-dependent relaxation, described by Furchgott and the others, is just one of the myriad mediator functions of NO. NO also carries important information in the nervous system: In the brain, NO has been shown to be a neurotransmitter, and plays an important role in learning and memory[6]. In male, it is a message that translates sexual excitement into an erect penis. Pfizer's blockbuster drug Viagra reverses impotence by enhancing a NO-stimulated pathway. At a time when vascular pharmacologists were pursuing the identity of EDRF, immunologists were independently exploring the cytotoxic properties of macrophages that appeared to be dependent on the generation of large amounts of NO in response to infection[7, 8]. The immune system uses NO in fighting virus, bacteria, parasites, and tumors. By early 1990's, scientists in distinct disciplines-immunology, cardiovascular physiology and neuroscience-suddenly realized they were studying the same molecule, NO.

Nitric oxide gene family

NO is a highly labile gas, and a very small compound that is not stored, but diffuses from its site of formation to its site of action (since it is both water/lipid soluble, it diffuses freely within tissues). Because it contains an unpaired electron, it is extremely active. It binds to the heme moiety of guanylate cyclase, and causes a greater than 400-fold activation of the enzyme. NO is formed directly from the guanidino nitrogen of the L-arginine by nitric oxide synthase(NOS) through a process that consumes five electrons, and results in the formation of L-citrulline (Fig 1). NOS is an unusual oxidative enzyme, in that most other enzymes consume only one or

two electrons for a similar function.

The structures and functions of NO synthases have been clarified by molecular cloning of the cDNA from the brain (NOS1)[9], endothelium (NOS3)[10], macrophages and other type of inducible cells (NOS2)[11] (Fig 2). Three forms of the NO synthase enzyme are known: NOS3 being initially isolated from the endothelium, NOS1 being isolated from brain cerebellum, and NOS2 being isolated from murine macrophages and human hepatocytes and chondrocytes. The molecular cloning, functional analysis and crystal structure study data revealed that NOS gene family shares similar compositions with each other. Comparison of the deduced sequence of the human inducible NO synthase (NOS2) with those of the two other human NO synthases shows 51% and 54 % identity and 68 % and 70 % similarity with the endothelial (NOS3) and neuronal (NOS1) NO synthase, respectively. These NOS enzymes all have two domains: N-terminal half of Heme-oxygenase domain with tetrahydrobiopterin, heme and arginine binding sites, and C-terminal half of P-450 reductase domain with the recognition sites for NADPH, as well as for flavin mononucleotide (FMN) and flavin adenosine dinucleotide(FAD). Cytochrome P-450 reductase is the only other mammalian enzyme known to contain these recognition sites (Fig 2).

Both NOS3, and NOS1 enzymes are constitutively expressed in the sense that their activation does not require new enzyme protein synthesis. For instance, the NOS1 is activated by glutamate-induced increase in intracellular Ca^{2+} level which in turn activates NOS via calmodulin. NOS3 is also a constitutive enzyme, being similarly activated by the increase in intracellular Ca^{2+} level. Agonists such as

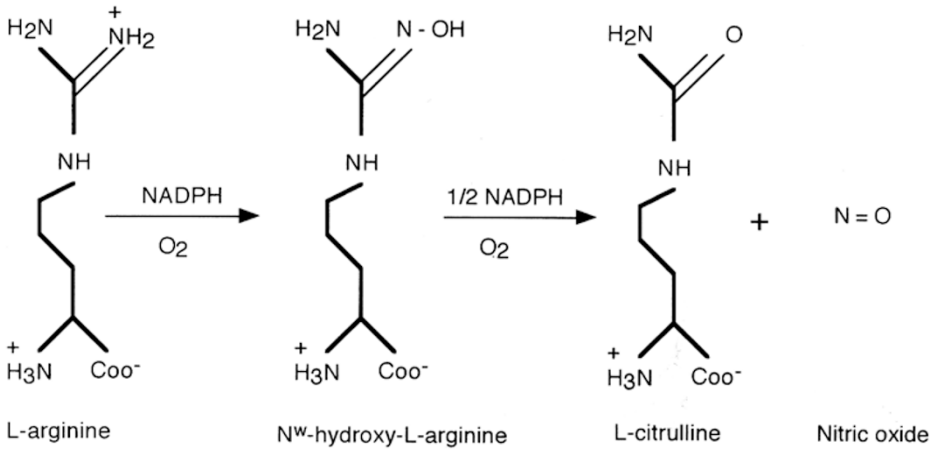

Fig 1. Biosynthesis of NO. L-arginine is converted to No in two successive steps ofwhich a two-electron oxidation of L-arginine to N^{w}-hydroxy-L-argninie is the first step, then converted to NO and citrulline, utilizing one and half NADPH and O2. Both steps require Ca^{2+} and calmodulin as activators and are accelerated by tetrahydrobiopterin.

acetylcholine and bradykinin activate muscarinic or bradykinin receptor on the endothelial cells to generate Ca^{2+} which then stimulates NOS through binding to calmadulin. Constitutive NOS enzymes account for the role of NO in mediating rapid events, such as neurotransmission, and blood vessel dilatation. In constrast, the inducible nitric oxide synthase, NOS2, which mediates the production of large amount of NO, can be activated by endotoxin and cytokines.

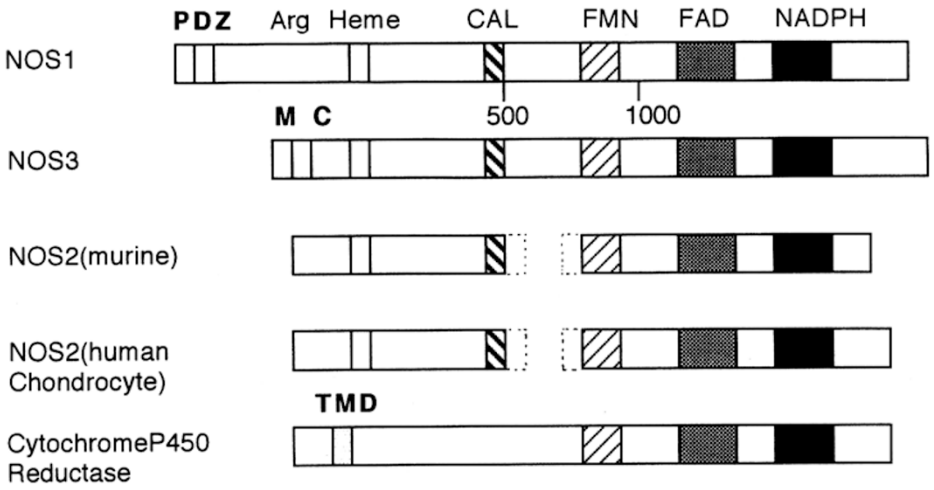

Fig 2. Sequence homologies of molecular isoforms of NOS. All NOS enzymes have consensus binding sites for Arginine (Arg), Heme, FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide), NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) and calmodulin (Cal). NOS1 has PDZ (Postsynaptic density protein, Disk large and Zo-1) binding domain. NOS3 has myristoylation (M) and Caveolin(C) binding sites although the site of Caveolin are only localized between amino acids 310 and 570. NOS2 cDNA, both NOS2 (murine) and NOS2 (human chondrocyte), is the shortest member of the family, lacking a 45-amino acid region (represented by the broken line) from the middle of their molecule. Cytochrome P450 reductase has transmembrane domain (TMD).

Recent progress in genetic study of NOS gene family

Soon after the first NOS has been cloned in 1991[9], we have mapped this gene on the human chromosome 12q12-q24 in 1992[12]. In 1993, the fluorescence in situ hybridization has been used to pinpoint the gene on 12q24.3[13], followed by identifying the highly polymorphic markers for NOS1 gene[14]. This was succeeded by establishing the linkage map of the gene location, which leads to the identification of the human disease association with NOS1 gene. It turns out NOS1 is a susceptibility locus for infantile pyloric stenosis (PS), one of the most common and lethal condition in new born babies. In the 27 family studies, there were significant overall transmission disequilibrium between PS and NOSla ($P < 0.006$)[15]. Coincidentally,

the most evident effect of disrupting the NOS1 gene in mice is the development of grossly enlarged stomach, with hypertrophy of the pyloric sphincter and the circular muscle layer[16]. This phenotype resembles the human disorder infantile pyloric stenosis, in which gastric outlet obstruction is associated with the lack of NADH neurons in the pylorus.

In order to hunt down the other human diseases in which NOS genes may be involved in, we and others also quickly carried out gene mapping studies, polymorphic marker scans, and disease association studies for the NOS2 and NOS3 genes. The NOS3 have been mapped to 7q35-q36 and NOS2 to 17q11.2[17]. We have discovered the NOS gene family is a dispersed gene family[18]. Although NOS1 and NOS3 genes have been shown to be only a single chromosomal localization, we have found multiple copies of inducible nitric oxide synthase gene-like sequences in the human genome. In human chromosome 17, there are several positive in situ hybridization signals on both arms of chromosome, spanning from 17p11.2 to 17q11.2[17]. So far, only NOS2A, which is located on 17q11.2, has been found to code for functional cDNAs. The other two NOS2-1ike sequences, NOS2B and NOS2C, have not been found to contain functional cDNAs (Tab 1)[19]. The genomic structures of the NOS1, NOS2 and NOS3 gene were also determined by our group and others (Tab 2)[20].

Tab 1. Chromosomal localization of NOS1, NOS3, NOS2 and NOS2-1ike sequences onto different regions of human chromosomes

Name	Other name	Type	Regulated by	Present in	Human chromossome
NOS1	Neuronal NOS nNO3	constitutive	Ca^{2+} / calmodulin	brain, cerebellum and other neuronal tissues	12q24.1-12q12.31
NOS3	Endothelium NOS, eNOS	constitutive	Ca^{2+} / calmodulin	endothelial cells	7q35-7q36
NOS2A	Inducible NOS iNOS	inducible	endotoxin cytokines	macrophages neutrophiles chondrocytes hepatocytes	17q11.2
NOS2B		unknown		unknown	17p11.2-17q11.2
NOS2C		unknown		unknown	17p11.2-17q11.2

As expected from physiological role of NOS3 gene, the NOS3 gene knock out mice has been shown to have phenotype of hypertension[21]. NOS2 knock out mice has been shown to be uniformly susceptible to infection by many pathogens, including tuberculosis[22, 23]. Therefore, it is one of the crucial genes to the host defence against many lethal pathogens. Recently, we have identified a highly polymorphic microsatellite marker in the human NOS2 promoter region[24], and found a strong positive allele association between human NOS2 gene polymorphism and fetal cerebra malaria[25]. Infectious diseases are still the major health risks in developing

countries. Future study of the NOS2 gene may help to unlock new methods to combat these diseases.

Tab 2. Comparison of the localization and size of exon/intron among NOS1, NOS2 and NOS3 genes (ref.20). 5'-UTR, 5'-untranslated region. 3'-UTR, 3'-untranslated region, bp, base pair. Several critical cofactor binding sites for NO synthase, including those for heme, Ca^{2+}calmodulin, FMN, FAD and NADPH are highly conserved among these three NOS genes. There are three types of the intron splice junctions. The junctions that found between codons are junction 0, that after the first nucleotide, junction I and that after the second nucleotide, junction II.

NOS2 Exon	Size bp	Feature	Intron (type)	NOS3 Exon(size)	Intron (type)	NOS1 Exon(size)	Intron (type)
1	192	5'UTR	1(II)				
2	183	5'UTR	2(II)				
3	85		3(0)				
4	123		4(0)				
5	149		5(II)				
6	163	Heme	6(0)	4(163)	4(0)	6(163)	6(0)
7	92		7(II)	5 (92)	5(II)	7 (92)	7(II)
8	142		8(0)	6(142)	6(0)	8(142)	8(0)
9	140		9(II)	7(140)	7(II)	9(140)	9(II)
10	175		10(0)	8(175)	8(0)	10(175)	10(0)
11	102		11(0)	9(109)	9(0)	11(102)	11(0)
12	195		12(0)	10(195)	10(0)	12(195)	12(0)
13	83	Ca^{2+} calmodulin	13(II)		11(II)	13(86)	13(II)
14	145	Ca^{2+} calmodulin	14(0)	12(145)	12(0)	14(145)	14(0)
15	105		15(0)	13(105)	13(0)	15(105)	15(0)
16	50		16(II)	15(117)	15(II)		16(II)
17	175	FMN binding	17(0)	16(175)	16(0)	18(175)	18(0)
18	133		18(I)		17(I)		19(I)
19	79		19(II)	18(79)	18(II)	20(79)	20(II)
20	182	FAD binding	20(I)		19(I)	21(194)	21(I)
21	164	FAD binding	21(0)		20(0)	22(170)	22(0)
22	208	FAD binding	22(I)	21(211)	21(I)	23(211)	23(I)
23	88		23(II)	22(88)	22(II)	24(88)	24(II)
24	122	NADPH binding	24(I)	23(122)	23(I)	25(122)	25(I)
25	149	NADPH binding	25(0)	24(149)	24(0)	26(149)	26(0)
26	195	NADPH binding	26(0)	25(195)	25(0)	27(195)	27(0)
27	606	3'-UTR				29(2160)	

Alfred Nobel was prescribed nitroglycerin, one of the key components of dynamite, to ease his chest pain when he contracted heart disease. It took 100 years until it was clarified that nitroglycerin acts by releasing nitric oxide gas. However, it also opened floodgate for medical articles detailing the biological activity of nitric oxide (NO) which are now flooding the scientific journals at a pace of 500 papers per month. There are more than 22,000 medical articles that dealt with nitric oxide, yet nitric oxide was not even a subject 12 years ago. It is impossible for any review to cover such a vast amount of information now. The only hope we have is that this rapid progress of Nitric Oxide Research will bring more benefit for human health.

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