British Journal of Pharmacology

British Journal of Pharmacology (2019) **176** 131–134 131

Themed Section: Nitric Oxide 20 Years from the 1998 Nobel Prize

EDITORIAL

Nitric oxide is not just blowing in the wind

Correspondence Louis J Ignarro, UCLA School of Medicine, Los Angeles, 269 South Beverly Drive, Unit 288, Beverly Hills, CA 90212, USA. E-mail: lignarro@gmail.com

Louis J Ignarro

UCLA School of Medicine, Beverly Hills, CA, USA

LINKED ARTICLES

This article is part of a themed section on Nitric Oxide 20 Years from the 1998 Nobel Prize. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v176.2/issuetoc

In 1998, Robert Furchgott, Louis Ignarro and Ferid Murad were awarded the Nobel Prize in Physiology or Medicine for 'for their discoveries concerning nitric oxide as a signalling molecule in the cardiovascular system'. Before these discoveries, **NO** was considered to be a caustic pollutant gas in the earth's atmosphere, with chemical properties that would preclude its consideration as an endogenous signalling molecule of any sort. For example, NO is highly lipid soluble, freely permeates all membranes and is a free radical readily reacting with oxygen and other radicals, iron and sulphur species. How could such a molecule exist endogenously, much less act as a signalling molecule? However, the discovery (Ignarro *et al.*, 1986a,b, 1987a,b) that mammalian tissues produce NO as a signalling molecule created an avalanche of studies by hundreds of investigators that established, unequivocally, the physiological relevance of the NO-**cyclic GMP** pathway. Interestingly, the very chemical properties that earlier argued against NO as a possible endogenous molecule turned out to be the most salient characteristics that make NO the perfect signalling molecule.

The earliest pharmacological experiments with NO were performed by Ferid Murad, who demonstrated in 1977 (Katsuki *et al.*, 1977) that NO gas could activate the cytosolic isoform of **guanylyl cyclase (GC),** thereby catalysing the conversion of **GTP** to cyclic GMP. This idea to test NO for such activity came from an astute observation by Murad, followed by his passion for mechanistic approaches to pharmacology. In working with enzyme protein solutions, bacterial contamination can often destroy the protein or reduce its catalytic activity. The antibacterial agent, sodium azide (NaN_3), was added to unpurified GC preparations to provide stability and extend catalytic activity. Murad noted that such azide-treated preparations had about 100-fold more catalytic activity than untreated enzyme (Mittal *et al.*, 1975). Moreover, addition of NaN_3 to isolated tissue preparations caused a marked increase in cyclic GMP levels. In a systematic series of experiments, it was found that azide, in the presence of catalase, was converted to NO, which accounted for the markedly higher GC activity and increased tissue levels of cyclic GMP (Katsuki *et al.*, 1977). These experiments with NaN_3 and NO launched the field of NO research.

Additional experiments revealed that certain chemicals containing NO or related groups could also elevate tissue levels of cyclic GMP, and if the tissue studied was nonvascular smooth muscle, relaxation was observed (Katsuki *et al.*, 1977). My laboratory revealed that vascular smooth muscle was also relaxed by NO, NO donor compounds, and that cyclic GMP was the second messenger mediating the effect of NO (Gruetter *et al.*, 1979). Even the more structurally complex organic nitrate and nitrite esters were demonstrated to elicit vascular smooth muscle relaxation by being metabolized to NO (Ignarro *et al.*, 1981).

The years 1980 and 1981 were pivotal years for basic research in the NO and vascular biology fields. NO was shown to be a potent inhibitor of human platelet aggregation, working through cyclic GMP mechanisms (Mellion *et al.*, 1981). **Nitroglycerin** was demonstrated unequivocally to promote vasodilation by NO-cyclic GMP mechanisms (Ignarro and Gruetter, 1980). S-Nitrosothiols were synthesized and shown to be excellent NO-donor compounds in the laboratory (Ignarro *et al.*, 1980; Ignarro and Gruetter, 1980). **ACh** was shown to require the presence of a healthy, functioning vascular endothelial cell layer to cause relaxation of the underlying smooth muscle (Furchgott and Zawadzki, 1980). This was the discovery of endothelium-derived relaxing factor or EDRF by Robert Furchgott.

Summarizing, three principal discoveries resulted in the awarding of the Nobel Prize in Physiology or Medicine for NO in 1998. Firstly, Ferid Murad discovered that NO activates GC and elevates tissue levels of cyclic GMP, thereby establishing the NO-cyclic GMP pathway. Secondly, Robert Furchgott discovered EDRF. Thirdly, my group discovered that EDRF is NO,

© 2018 The British Pharmacological Society

DOI:10.1111/bph.14540

thereby establishing that mammalian cells produce NO. Since the discovery that EDRF is NO in 1986, literally tens of thousands of papers were published on the chemistry and biology of NO during the subsequent 12 year period leading up to the Nobel Prize. I know of no other field of biomedical research that exploded in such an astonishing manner over such a short period of time. The interested reader is referred to Volume 1 of 'Nitric Oxide – Biology and Pathobiology' (Ignarro, 2000).

One additional discovery that is pertinent to NO and cyclic GMP, which is worth mentioning here, is the work that culminated in the development and marketing of Viagra^R (**sildenafil**) for the treatment of erectile dysfunction. NO was reported to be the principal neurotransmitter of the NANC neurons innervating the corpus cavernosum or erectile tissue in rabbits (Ignarro *et al.*, 1990) and humans (Rajfer *et al.*, 1992). The Pfizer Pharmaceutical Company followed up on these observations and developed a drug (sildenafil) for the treatment of erectile dysfunction in men. Sildenafil works as a potent selective PDE inhibitor (**PDE-5**, an isoform that is concentrated in erectile tissue) and results in the marked enhancement of tissue cyclic GMP accumulation in response to only small amounts of NO. The marketing of sildenafil as Viagra^R took place in March of 1998. Interestingly, just 7 months later, the announcement was made for the 1998 Nobel Prize in the field of NO.

In this themed section, which celebrates 20 years since the 1998 Nobel Prize, we have an opportunity to see how much further the field has advanced since that enlightening day in Stockholm. The discovery of NO as an endogenously generated effector molecule was a paradigm shift in biological signalling and suggested the possibility that other small, freely diffusible molecules might be synthesized endogenously as active molecules. Such other molecules included HNO, **CO** and **H_2S**. In this issue, Fukuto discusses the discovery of HNO (nitroxyl) as an important, potential signalling molecule in the mammalian species (Fukuto, 2019). HNO possesses unique effects on the heart, namely, a positive inotropic effect that is distinct from the effects of NO. Therefore, HNO or HNO donor compounds represent a novel and pharmacologically important strategy for treating heart failure. Due to the recognition of the therapeutic potential and clinical demand for a drug to treat heart failure, clinical studies using HNO donors show encouraging results (Tita *et al.*, 2017).

As in many other tissues, NO plays key roles in regulating a wide range of functions in the gastrointestinal tract in both health and disease, such as maintenance of mucosal integrity, through modulation of mucosal defence and through regulation of secretion and smooth muscle function. NO also plays a role in pathophysiology, particularly with respect to regulating mucosal inflammation, enteric pain and responses to injury. There are many poorly managed diseases of the GI tract, such as inflammatory bowel disease, for which NO-based therapies hold significant promise. In this issue, Wallace (2019) reviews the contributions of NO to mucosal defence and disease, with examples provided of attempts to develop NO-based treatments for some prevalent GI disorders.

It has become increasingly evident during the past decade that the efficacy of cancer therapy depends not only on the ability to successfully target tumour cells but also activation of the immune system to control tumour progression. NO generated by **NOS-2** and **PGE_2** generated by **COX-2** have emerged as key players in the regulation of cytokine-mediated signalling and cellular metabolism. Both enzymes are inducible rather than constitutively expressed. Triple negative breast cancer (TNBC) is an inflammation-driven and very aggressive cancer. Therefore, it is not surprising that co-expression of high NOS-2 and COX-2 leads to dramatically reduced patient survival. Here, we have a clear situation where two distinct signalling molecules, NO and PGE_2, when produced in relatively large quantities, can exacerbate certain forms of breast cancer. This phenotype is supported by feed-forward mechanisms where NOS-2 is activated by PGE_2, and COX-2 is activated by NO, and these pathways involve cytokines such as **IL-8 (CXCL8), IL-6** and **TNF-α**. In this issue, Basudhar *et al.* discuss how further insights into the interface between metabolism and immunity can lead to novel therapeutic approaches by inhibiting the overproduction of both NO and PGE_2 (Basudhar *et al.*, 2019).

The studies involving NOS have played a prominent role in NO biology and pathobiology from its discovery to the present time. Literally, thousands of research articles have been published on the isoforms of NOS. The discovery of NOS was made by Bredt and Snyder in 1990, approximately 4 years after NO was first shown to be produced in mammalian tissues. This particular NOS was from rat cerebellum and, therefore, was the **neuronal NOS isoform (nNOS) or NOS-1**. In this first and historic article, not only was the enzymatic biosynthesis of NO revealed but the enzyme was isolated, purified, characterized (requiring NADPH and calcium; producing stoichiometric quantities of **citrulline** as a second reaction product) and shown to be dependent on the presence of calmodulin for catalytic activity (Bredt and Snyder, 1990). The discovery of constitutively expressed neuronal NOS was quickly followed by the identification of **endothelium-derived NOS (eNOS; NOS-3**; constitutively expressed) and inducible NOS (iNOS; NOS-2; not constitutively expressed). In this issue, Stuehr and Haque discuss what was known about NOS enzymology before the 1998 Nobel Prize and what has been learned since that time (Stuehr and Haque, 2019). The more recent advances made include NOS maturation and assembly, with particular emphasis on the haem insertion process and catalytic feedback effects of NO itself.

Knowledge of the pharmacology of NO in the cardiovascular system by 1980, coupled with the discovery in 1986 that vascular endothelial cells produce NO, led to the inevitable discovery of endothelial NOS or eNOS (Förstermann *et al.*, 1991). This discovery of a unique membrane-bound isoform of NOS subsequently led to an explosion of mechanistic information on the molecular and cellular pathways that regulate eNOS function and catalytic activity. Moreover, these studies allowed the systematic exploration of how this enzyme is regulated in health and disease. As Garcia and Sessa (2019) discuss in this issue, eNOS is critical for normal vascular homeostasis, vascular remodelling and adaptation to stress and exercise. Central to this field of study is whether correction of impaired endothelium-dependent responses, which is the hallmark of cardiovascular disease progression, would delay or prevent disease. Answers to such critically important questions will inevitably come from a deeper understanding of the multifaceted regulation of eNOS and should lead to novel therapeutic approaches for the prevention and/or treatment of vascular dysfunction and disease.

Since the early measurements of cyclic GMP levels in mammalian tissues, it was appreciated that the brain,

particularly the cerebellum, had relatively high levels. In the late 1970s, NO was shown to stimulate the biosynthesis of cyclic GMP in numerous tissues including brain. Interestingly, more than 10 years before the discovery of neuronal NOS, the addition of **L-arginine** to brain tissue was found to increase the levels of cyclic GMP. This was later shown to be attributed to the conversion of L-arginine to NO by nNOS, and subsequent activation of cytosolic GC by NO. In this issue, Garthwaite (2019) provides a detailed and exciting account of the chronology of experiments leading to the identification of NO as an important neuronal transmitter in the brain (Garthwaite *et al*., 1988). These studies show clearly how seemingly diverse experimental results emerging from different laboratories can converge into the discovery of a distinct and novel CNS neurotransmitter. This discovery accounts for the present-day knowledge of high levels of nNOS, soluble GC and cyclic GMP in brain tissue. Moreover, we now have a much better idea of the functions of NO and cyclic GMP in certain regions of the brain. As Garthwaite highlights in this issue, the past 20 years have been characterized by major changes in our understanding of the NO-cyclic GMP signalling pathway, both in terms of its overall effects on many different overall brain functions and in the details of how it operates.

At about the time NO was identified as the NANC neurotransmitter responsible for penile erection (Ignarro *et al*., 1990), NO was demonstrated to function as an inhibitory neurotransmitter in the enteric nervous system (Bult *et al*., 1990). Soon thereafter, other evidence amassed that established NO as an enteric inhibitory neurotransmitter. As a consequence, the terminology rapidly changed to describe responses mediated by NO from NANC neurons as nitrergic; it was no longer necessary to refer to such responses as NANC. In this issue, Sanders and Ward focus on the role and mechanisms of NO as an enteric neurotransmitter that have emerged since the Nobel Prize for NO in 1998 (Sanders and Ward, 2019). It is clear that nitrergic regulation is extremely important in GI motility, as NO is a major inhibitory neurotransmitter in nearly every region, and is a mediator of inflammatory effects during overproduction.

Soon after the discoveries that NO is produced in mammalian cells and that the mechanism of NO production is *via* the catalysed oxidation of L-arginine by NOS, attention was focused on the mechanisms by which the physiological effects of NO are terminated. NO undergoes numerous and distinct oxidation reactions to other nitrogen oxides, which either are biologically inactive or do not share the same biological activity as NO itself. Perhaps the most prevalent oxidation reaction in biologic tissues is the conversion of NO to NO_2^- (nitrite) and NO_3^- (nitrate). NO_2^- and NO_3^- are often thought of as being the inactive metabolic products of NO since the biological activity of NO_2^- is several orders of magnitude lower than NO, and NO_3^- is essentially inactive. This concept was dogma for many years until more recent observations showed clearly that both NO_2^- and NO_3^- are biologically active, albeit indirectly. In this issue, DeMartino *et al*. discuss the progression of studies demonstrating that NO_2^- and NO_3^- serve as biological reserves of NO, and under what conditions NO can be produced from such stores (DeMartino *et al*., 2019). Today, it is clear that NO_2^- and NO_3^- are not simply inactive products of NO metabolism but play important roles in conserving NO. The fact that the products of NO biosynthesis and metabolism all serve as biological reserves of NO is intriguing. The second reaction product of the NOS pathway is L-citrulline, which is enzymically recycled back to L-arginine, the substrate for NOS to produce more NO. Likewise, NO_2^- and NO_3^-, the oxidation products of NO, act as reserves of NO because each can be converted back to NO by distinct reductive pathways. The biological importance and potential clinical application of these concepts are nicely discussed in this issue.

The first clinical application of NO gas was pioneered by Warren Zapol in the early 1990s at the Massachusetts General Hospital in Boston. In this issue, Yu *et al*. describe the initial experiments performed in awake sheep with pulmonary hypertension, and how this led to clinical studies in infants suffering from persistent pulmonary hypertension (Yu *et al*., 2019). Initially, some thought that inhaled NO would be toxic to animals, including humans, because of the oxidation of NO in air to NO_2 (nitrogen dioxide) gas, which is highly toxic. However, the chemistry of NO is kind to the inhaler of small quantities because the reaction between NO and O_2 is second order in NO and first order in O_2. That is, high concentrations of NO react with O_2 at a very fast rate to produce high concentrations of NO_2. However, very low concentrations of NO (e.g. 10 p.p.m. or less) react very slowly with O_2 to form negligible quantities of toxic NO_2. Therefore, small quantities of NO can be mixed with O_2 and safely administered to animals and humans by inhalation, without any significant presence of NO_2. As a safety measure, the inhaled NO/O_2 gas mixture is scrubbed in the apparatus just prior to inhalation in order to remove any small amounts of NO_2 that might be formed. The inhaled NO reaches the patent airways and diffuses into the adjacent pulmonary arteries to dilate them and alleviate pulmonary hypertension by decreasing pulmonary artery pressure and resistance. The vasodilation is specific for the pulmonary vasculature because any NO that gets into the circulation is immediately destroyed by oxyhaemoglobin, thereby preventing any downstream systemic vasodilation. Inhaled NO therapy affords a safe and non-invasive method of treating persistent pulmonary hypertension in newborn babies. In this issue, the many advances made in the use of inhaled NO to treat a variety of cardio-pulmonary disorders are discussed. In addition, a novel and economic method of production of NO, which uses pulsed electrical discharges in air to produce therapeutic levels of NO that can be used for inhalation therapy, is described in detail.

This themed section provides a snapshot of the progress that has been made in the field of NO research during the past 20 years. This begs the question of what more will we understand 20 years from now? In my view, the chemical biology of NO strongly suggests, if not indicates, that many additional physiological actions will be discovered. Just as one example, the complex interactions between NO and haem-containing proteins are likely to uncover a host of new regulatory mechanisms and pathways. Another example is the physiological significance of the relatively high levels of neuronal NOS and cyclic GMP above and beyond their known roles in neurotransmission and neuromodulation. Finally, the widespread interactions between NO and ubiquitous sulphur-containing molecules are likely to uncover new signal

transduction mechanisms by which NO regulates a host of diverse biological pathways.

Conflict of interest

The author is owner of, and receives compensation from, a commercial establishment specializing in the development of nutritional supplements that stimulate nitric oxide production and action.

References

Basudhar D, Bharadwaj G, Somasundaram V, Cheng RYS, Ridnour LA, Fujita M *et al.* (2019). Understanding the tumour microenvironment communication network from an NOS2/COX2 perspective. Br J Pharmacol 176: 155–176.

Bredt DS, Snyder SH (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc Natl Acad Sci U S A 87: 682–685.

Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH, Van Maercke YM, Herman AG (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. Nature 345: 346–347.

DeMartino AW, Kim-Shapiro DB, Patel RP, Gladwin MT (2019). Nitrite and nitrate chemical biology and signalling. Br J Pharmacol 176: 228–245.

Förstermann U, Pollock JS, Schmidt HHHW, Heller M, Murad F (1991). Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. Proc Natl Acad Sci U S A 88: 1788–1792.

Fukuto JM (2019). A recent history of nitroxyl chemistry, pharmacology and therapeutic potential. Br J Pharmacol 176: 135–146.

Furchgott RF, Zawadzki JV (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288: 373–376.

Garcia V, Sessa WC (2019). Endothelial NOS: perspective and recent developments. Br J Pharmacol 176: 189–196.

Garthwaite J (2019). NO as a multimodal transmitter in the brain: discovery and current status. Br J Pharmacol 176: 197–211.

Garthwaite J, Charles SL, Chess Williams R (1988). Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature 336: 385–388.

Gruetter CA, Barry BK, McNamara DB, Gruetter DY, Kadowitz PJ, Ignarro LJ (1979). Relaxation of bovine coronary artery and activation of coronary arterial guanylyl cyclase by nitric oxide, nitroprusside and a carcinogenic nitrosoamine. J Cyclic Nucleotide Res 5: 211–224.

Ignarro LJ (ed) (2000). Nitric Oxide – Biology and Pathobiology. Academic Press: San Diego.

Ignarro LJ, Gruetter CA (1980). Requirement of thiols for activation of coronary arterial guanylyl cyclase by glyceryl trinitrate and sodium nitrite: possible involvement of S-nitrosothiols. Biochim Biophys Acta 631: 221–231.

Ignarro LJ, Barry BK, Gruetter DY, Edwards JC, Ohlstein EH, Gruetter CA *et al.* (1980). Guanylyl cyclase activation by nitroprusside and nitrosoguanidine is related to formation of S-nitrosothiol intermediates. Biochem Biophys Res Commun 94: 93–100.

Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G (1987b). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci U S A 84: 9265–9269.

Ignarro LJ, Bush PA, Buga GM, Wood KS, Fukuto JM, Rajfer J (1990). Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. Biochem Biophys Res Commun 170: 843–850.

Ignarro LJ, Byrns RE, Wood KS (1986a). Pharmacological and biochemical properties of endothelium-derived relaxing factor (EDRF): evidence that EDRF is closely related to nitric oxide (NO) radical. Circulation 74: II–287.

Ignarro LJ, Byrns RE, Buga GM, Wood KS (1987a). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacological and chemical properties that are identical to those for nitric oxide radical. Circ Res 61: 866–879.

Ignarro LJ, Harbison RG, Wood KS, Kadowitz PJ (1986b). Activation of purified soluble guanylyl cyclase by endothelium-derived relaxing factor from intrapulmonary artery and vein: stimulation by acetylcholine, bradykinin and arachidonic acid. J Pharmacol Exp Ther 237: 893–900.

Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Hyman AL, Kadowitz PJ *et al.* (1981). Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. J Pharmacol Exp Ther 218: 739–749.

Katsuki S, Arnold W, Mittal C, Murad F (1977). Stimulation of guanylyl cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. J Cyclic Nucleotide Res 3: 23–35.

Mellion BT, Ignarro LJ, Ohlstein EH, Pontecorvo EG, Hyman AL, Kadowitz PJ (1981). Evidence for the inhibitory role of guanosine 3′,5′-monophosphate in ADP-induced human platelet aggregation. Blood 57: 946–955.

Mittal CK, Kimura H, Murad F (1975). Requirement for a macromolecular factor for sodium azide activation of guanylyl cyclase. J Cyclic Nucleotide Res 1: 261–269.

Rajfer J, Aronson WJ, Bush PA, Dorey FJ, Ignarro LJ (1992). Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. N Engl J Med 326: 90–94.

Sanders KM, Ward SM (2019). Nitric oxide and its role as a non-adrenergic, non-cholinergic inhibitory neurotransmitter in the gastrointestinal tract. Br J Pharmacol 176: 212–227.

Stuehr DJ, Haque MM (2019). Nitric oxide synthase enzymology in the 20 years after the Nobel Prize. Br J Pharmacol 176: 177–188.

Tita C, Gilbert EM, Van Bakel AB, Grzybowski J, Haas GJ, Jarrah M *et al.* (2017). A Phase 2a dose-escalation study of the safety, tolerability, pharmacokinetics and haemodynamic effects of BMS-986231 in hospitalized patients with heart failure with reduced ejection fraction. Eur J Heart Fail 19: 1321–1332.

Yu B, Ichinose F, Bloch DB, Zapol WM (2019). Inhaled nitric oxide. Br J Pharmacol 176: 246–255.

Wallace JL (2019). Nitric oxide in the gastrointestinal tract: opportunities for drug development. Br J Pharmacol 176: 147–154.