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Nitrogen: The unsung hero of vascular physiology

IN DAY-TO-DAY MEDICAL PRACTICE, the seventh element on the periodic table—nitrogen—may not come to mind often. But it is more exciting than you might think. In fact, there is an entire nitrogen cycle that you probably learned but forgot about to make room for the much more popular carbon and water cycles.

Associate Editor Adam Brown, MD, discusses an angle related to the article "Should I start anticoagulation in my patient newly diagnosed with pulmonary hypertension?" on page 339.

Nitrogen is all around us. It makes up 80% of our atmosphere, and we have learned to extract it from the air and inject it into our soil (known as *nitrogen fixation*) to grow crops. Nitrogen is a component of amino acids and of DNA and RNA, and it's crucial for protein synthesis. Inhaling certain forms of nitrogen leads to a stumbling gait and fits of laughter, yet nitrogen in another form is a common explosive.

What may *not* be appreciated is the importance of the nitrogen compound nitric oxide in vascular physiology. An article in this issue of the *Journal* presents a question about starting a patient newly diagnosed with pulmonary hypertension on anticoagulants, but therapeutic options can also include medications that manipulate a tissue's response to nitric oxide.[1] The journey to understand nitric oxide's role in vasodilation, and how it could be used therapeutically, had many stumbling blocks along the way, but a combination of discoveries eventually led to breakthroughs in treating angina, erectile dysfunction, and pulmonary arterial hypertension.

THE NERVES AND VESSELS

Our nitric oxide journey must start with understanding the link between nerves and blood vessels. In the 19th century, the French physiologist Dr. Claude Bernard[2] performed experiments by severing the cervical sympathetic ganglion in rabbits, which resulted in increased "calorification" (heat production) and vasodilation (widening of visible vessels in the thin skin of an albino rabbit's ear). This was the first clear demonstration of neural regulation of blood vessel physiology.[3] Decades would pass, during which there was much controversy and arguing among neurologists, before we understood *how* the nerves influence vasodilation or vasoconstriction—was it electricity or some kind of neurotransmitter?

The hormone adrenaline was discovered in 1894 and found to mimic the sympathetic nervous system, triggering vasoconstriction and elevating blood pressure.[4] Acetylcholine, a neurotransmitter, was later discovered to be a potent vasodilator, but the mystery of its full involvement in physiology took much longer to unravel because acetylcholine was difficult to detect in tissues. Acetylcholine is tightly regulated, so it is rapidly broken down by acetylcholinesterase on release from the nerve. The breakthrough came by using eserine, an extract from the Calabar bean and a known neurotoxin, which inhibited acetylcholinesterase and prevented acetylcholine from breaking down.[4] Once acetylcholine could be measured, its function as a key mediator of the parasympathetic nervous system, including lowering blood pressure by vasodilation, was quickly recognized.

NITRIC OXIDE'S LINK TO ACETYLCHOLINE

In 1976, Furchgott and Zawadzski[5] used a bioassay and tissue culture to understand the mechanism of *how* acetylcholine interacts with vascular smooth muscle to cause relaxation. Metal probes inserted into the lumen of a rabbit aorta measured the force exerted

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from the smooth muscle contracting or dilating against the probes when the aorta was exposed to various substances such as histamine, serotonin, angiotensin, and acetylcholine. The 2 doctors recognized a problem: when the isolated rabbit aorta was exposed to acetylcholine, no relaxation occurred. In the process of preparing the tissue, filter paper was used to rub the endothelial cells off the lumen of the aorta to allow acetylcholine direct access to the smooth muscle. They repeated the experiment multiple times until finally trying the experiment *without* rubbing away the endothelial lining, and voilà! The rabbit aorta dilated on contact with acetylcholine.[5]

The discovery that endothelial cells were important to vasodilation was critical. It would later be found that acetylcholine activates the formation of nitric oxide, as a gas, within endothelial cells, which is then diffused out of the cells and into the neighboring smooth muscle, triggering additional second messengers (eg, cyclic guanosine monophosphate and cyclic adenosine monophosphate) and smooth muscle relaxation.[6] This was a major achievement in understanding vascular physiology.

THERAPEUTIC USE OF NITROGEN AND TROUBLE WITH TACHYPHYLAXIS

As our understanding of nitric oxide's role in vasodilation evolved, treatment of hypertension was the obvious medical application, but there was a catch. Nitrogen-based compounds were used therapeutically long before we knew the role nitric oxide played in vasodilation. In the middle 19th century, nitroglycerin (which gets broken down to nitric oxide) began to be used in patients with anginal chest pain.[7] It's not clear why nitroglycerin was chosen to treat angina, but it's possibly because nitroglycerin ingestion caused tachycardia and thus had a clear physiologic effect on the heart.[6] However, a major limitation of therapeutic nitrogen was recognized very early: tachyphylaxis. In the early days of treating angina, a doctor noted his patient's chest pain responded to inhaling 5 to 10 drops of nitrite from a cloth, but efficacy waned with continued use, and the patient required increased doses to have the same response.[7] It became clear that, if nitrogen compounds were given continuously, patients rapidly developed a tolerance.

At the dawn of the 20th century, workers in trinitrotoluene factories were also aware that constant exposure to nitrate-containing compounds led to tachyphylaxis.[7] Workers often complained of headaches and a racing heart on Monday, and their symptoms would slowly resolve over the course of the week. Nitrate tolerance is short-lived, so after a day or 2 off on the weekends, symptoms would start again on Monday. This phenomenon was referred to as *Monday disease*.[7] It became practice for some workers to take home pieces of nitrate over the weekend to rub on their skin until returning to work on Monday, continuing the exposure and preventing the headaches they experienced when returning to work.[7–9]

CIRCUMVENTING TACHYPHYLAXIS AND THE BREAKTHROUGH

Like most things in medicine, overcoming nitrogen tolerance is complicated because nitric oxide doesn't act alone. Nitric oxide stimulates smooth muscle relaxation and vasodilation, but a series of second messengers are also triggered once nitric oxide diffuses into the smooth muscle cell, leading to decreased calcium levels and smooth muscle relaxation.[6] Given that tachyphylaxis develops in response to exogenous nitric oxide, could the second messengers, instead of nitric oxide, be manipulated to increase vasodilation and bypass tachyphylaxis?

In the middle 1980s, Pfizer's cardiovascular research division was looking for a novel target to treat hypertension and chose phosphodiesterase type 5 (PDE5).[6] PDE5 breaks down the second messengers responding to nitric oxide, decreasing the vasodilatory response. The goal was to *inhibit* PDE5, thus allowing the continuation of smooth muscle response to nitric oxide. Sildenafil was developed with hopes of treating hypertension and angina through PDE5 inhibition. The results of the initial trials are widely known in the medical world—men on the PDE5 inhibitor noted the development of erections.[6] The pursuit of sildenafil as a treatment for angina or hypertension was sidelined, and it became a blockbuster medication to treat erectile dysfunction.

THE PULMONARY ARTERIAL HYPERTENSION CONNECTION

Research on sildenafil provided evidence that not all vascular physiology is the same. The effect of PDE5 inhibitors on lowering *peripheral* blood pressure was modest, but certain tissues, such as the corpus cavernosum of the penis, have a profound response to the drug.[6] Further research explored the role of PDE5 in vascular territories throughout the body. Using a combination of animal models and human tissue, a particularly high expression of PDE5 was found in lung tissue.[6,10] Nitric oxide turns out to be an important regulator of oxygenation and blood flow (ventilation-perfusion matching)

in the pulmonary vessels. As alveoli are aerated and expand, vascular endothelial cells are stretched and release nitric oxide, leading to vasodilation and increased blood flow to the well-oxygenated alveoli.[9] With a clearer sense of the roles nitric oxide and PDE5 play in pulmonary physiology, attention turned once again to treating pulmonary arterial hypertension with sildenafil.

Experiments with a chronically hypoxic rodent model demonstrated that treatment with a PDE5 inhibitor protected the mice from developing pulmonary hypertension.[6] Soon, multiple case reports were published on the efficacy of PDE5 inhibition in patients with pulmonary arterial hypertension, resulting in the SUPER-1 (Sildenafil Use in Pulmonary Hypertension) trial in 2002[11] that showed improvements in the 6-minute walk as well as pulmonary hemodynamics. Based on these favorable outcomes, the US Food and Drug Administration approved sildenafil in 2005 for the treatment of pulmonary arterial hypertension. Multiple medications are now approved to treat pulmonary arterial hypertension, including 2 PDE5 inhibitors.

CONCLUSION

Any farmer will proclaim the benefits of nitrogen in soil, but not every clinician can explain why nitrogen is critical to understanding vascular physiology. Nitric oxide's role in vasodilation was revealed because of a series of experiments and leaps in knowledge over the 19th and 20th centuries. The first was the discovery of the Calabar bean's importance in measuring and understanding acetylcholine's role in vasodilation.[4] Then the physical manipulation of the vascular lumen led to the recognition that a gas (nitric oxide) communicates between endothelial cells and vascular smooth muscle.[5,6] Further trial and error demonstrating nitric oxide's limitations as a therapeutic agent inspired the idea to manipulate second messengers with PDE5 inhibitors to circumvent tachyphylaxis.[6] Clinical use of these PDE5 inhibitors provided evidence that they target vessels in specific tissues, finally leading to a breakthrough in pulmonary arterial hypertension management.[6,10,11] The journey of nitric oxide's role in physiology shows the many steps required to develop a new therapeutic, as well as what a therapeutic can then teach us about normal physiology.

Next time you're looking at the periodic table of the elements, focus on atomic number 7, take a deep breath and hold it, feel those alveoli stretch, and appreciate the work of the vascular endothelial cells and the burst of nitric oxide.

DISCLOSURES

Dr. Brown has disclosed consulting and teaching and speaking for Amgen and Chemocentryx.

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