The excitement and rewards of research with our discovery of some of the biological effects of nitric oxide

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The rewards of biomedical research can be very exciting and remarkable. The feeling of having done an experiment for the first time and only you know the results that will have an important effect on your research and perhaps broader applications is exhilarating. I suspect that it is not common for scientists to see their fundamental research develop during their research career into such broad clinical application for numerous diseases. While much of our work and experiments were well planned, many of our important observations came from luck or serendipitous events—undoubtedly, a common phenomenon in biomedical research. I will recount some of these experiments here. A more detailed review can be found elsewhere.

I spent my early years as a trainee working with cAMP and adenylyl cyclase. As a result of this experience, after the discovery of cGMP in rat urine in the mid-1960s, I and others thought that cGMP might also be a second messenger to mediate the effects of some hormones and drugs. We transferred much of our work from cAMP to cGMP.

Our earliest experiments with guanylyl cyclase in the early 1970s suggested that there were at least two isoforms of the enzyme, soluble and particulate forms, with different kinetic properties. This was of interest since adenylyl cyclase was exclusively particulate. The possibility of soluble and particulate isoforms of guanylyl cyclase suggested that different classes of hormones might regulate these different enzymes to produce separate pools of cGMP that may have different physiological effects. This was indeed the case from our later work with different activators of each isoform as discussed below. We ultimately demonstrated there were different isoforms with purification, cloning, and expression some years later. However, we initially took a shortcut and added various inhibitors of nucleotidases, phosphatases, and phosphodiesterases to soluble and particulate preparations to see if their kinetic behaviors in crude preparations could be altered. Perhaps the kinetic differences of the apparent isoforms could be spurious due to nucleotidases and phosphatases competing for the GTP substrate or phosphodiesterases hydrolyzing our product cGMP. We added fluoride, pyrophosphate, azide, hydroxylamine, nitrite, methylxanthines, and other agents to our incubations. Several hormones that increased cGMP accumulation in intact tissues failed to activate the enzymes in cell-free preparations.

One of the goals of the laboratory was to understand the molecular mechanism of hormonal regulation of guanylyl cyclase. Without an effect of hormones in cell-free preparations, this would be a most difficult task. Quite accidentally we found that azide, hydroxylamine, and nitrite activated the soluble guanylyl cyclase. What an exciting event this was, and we became committed to understanding their mechanism of activation. We thought that these activators could help us reconstitute activation by some hormones in cell-free systems; after all, fluoride activation of adenylyl cyclase had proven useful in understanding hormonal activation of that enzyme.

The activation by azide was oxygen-dependent, increased by thiols, was tissue-specific, and demonstrated a time lag of several minutes before the rate of the activation was maximal. We reasoned that these activators were converted to another activating substance. Furthermore, we found that some heme-containing proteins, catalase, peroxidase, cytochromes, were required for activation by azide, and hemoglobin or myoglobin caused inhibition.

These activators also increased cGMP levels in several tissues. When we added azide, hydroxylamine, or nitrite to tracheal smooth muscle preparations, cGMP levels were increased as expected, and the tissue relaxed. It was quite a coincidence that we had tracheal smooth muscle preparations in the laboratory at the time. We developed this preparation of relatively homogeneous smooth muscle because I thought cGMP might cause smooth muscle contraction. We wanted to correlate the accumulation of cAMP and cGMP in homogeneous smooth muscle with contraction and relaxation. We initially avoided working with heterogenous smooth muscle preparations such as blood vessels, since we would be unable to identify which cells accumulated the cyclic nucleotides to affect the smooth muscle directly or indirectly. The opposite was true: cGMP accumulated with azide, hydroxylamine, or nitrite and phosphodiesterase inhibitors to cause relaxation of the preparations. We then tested other smooth muscle relaxants such as nitroglycerin and nitroprusside and found that they also activated soluble guanylyl cyclase and increased cGMP levels in several smooth muscle tissues (trachea and intestine) as well as many other tissues.

The dose-response and time course experiments convinced us that cGMP accumulation was associated with smooth muscle relaxation. Subsequently, we found that cGMP accumulation caused cGMP-dependent protein kinase activation, altering phosphorylation of a number of proteins and dephosphoryla-

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tion of myosin light chain.\textsuperscript{1,2} It was obvious that these cardiovascular drugs were converted to nitric oxide that activated guanylyl cyclase. We had figured out the mechanism of action of some important widely used drugs. Nitroglycerin had been used clinically since the 1870s for angina pectoris with no understanding of its mechanism of action. We coined the term “nitrovasodilators” for these nitric oxide prodrugs. We found that nitric oxide would activate all preparations of soluble guanylyl cyclase from most tissues examined.\textsuperscript{13} We found that nanomolar concentrations activated purified enzyme, and higher concentrations could activate as much as 200-fold.

We postulated that nitric oxide would be a ubiquitous intracellular agent to mediate the effects of various hormones on cGMP synthesis, ie, nitric oxide would function as a messenger.\textsuperscript{10,14} Because of the low tissue levels of nitric oxide, it took us another 8 years to prove this. In 1986, we developed a reporter cell assay for endothelial-derived relaxant factor (EDRF), EDRF-like substances, and nitric oxide.\textsuperscript{15} We found that numerous cell types released an EDRF-like/nitric oxide–like substance.\textsuperscript{16} The cell types and tissues included endothelial cells, kidney epithelial cells, neuroblastoma cells, and adrenal tissue. We began to consider EDRF an “endogenous nitrovasodilator.”\textsuperscript{17} We thought that EDRF was a nitro or nitroso adduct or complex with a thiol, lipid, or protein that could release nitric oxide.\textsuperscript{18} Shortly after this review article,\textsuperscript{17} Furchgott and Ignarro reported that EDFR was nitric oxide.

Another bit of serendipity relates to our work with atriopeptins (atrial natriuretic factor). In the late 1970s, we found that heat stable enterotoxin (ST) from some strains of \textit{Escherichia coli} activated the particulate isoform of guanylyl cyclase in intestinal mucosa. ST also increased cGMP levels in intestine that led to fluid and electrolyte secretion into the intestinal lumen to cause diarrhea.\textsuperscript{19} We thought that an endogenous peptide might exist to mimic the enterotoxin. Our attempts to extract an activity from several tissues were unsuccessful.

However, in the early 1980s, De Bold reported on the characterization of a new peptide hormone from heart, atrial natriuretic factor (ANF), that dilated blood vessels and caused natriuresis. ANF had some of the effects of nitrovasodilators and of \textit{E coli} enterotoxin. We found that ANF also activated particulate guanylyl cyclase, which was a receptor for ANF.\textsuperscript{20,21} Subsequently, a family of natriuretic peptides (ANP, CNP, BNP) were described, all of which activated particulate guanylyl cyclase. Later, another class of peptides, guanylin and uroguanylin, were described that also activated the particulate guanylyl cyclase. Thus, different classes of hormones could result in the activation of either soluble guanylyl cyclase or particulate guanylyl cyclase, as we suspected might be the case when we found the two different isoforms in 1974 to 1975.\textsuperscript{3,4} The particulate isoform is the receptor for several peptides.\textsuperscript{21,22}

In the late 1980s, the fields of nitric oxide and cGMP research exploded with greater and greater interests from more laboratories. The nitric oxide synthases that catalyze nitric oxide synthesis from arginine were purified, characterized, and cloned by several laboratories. The x-ray structures of their fragments have since become available. Numerous inhibitors have been prepared to the three isoforms, some of which have some selectivity for an isoform. Knockouts of each isoform have also been developed.

In the past decade, a number of clinical trials have been conducted. Inhaled nitric oxide gas has been approved for pulmonary hypertension in children. It has also been examined in premature babies with pulmonary hypertension, children with congenital heart disease, and adults with pulmonary hypertension and acute respiratory distress syndrome. Nitric oxide synthase inhibitors and/or nitric oxide scavengers have been studied in patients with septic shock, inflammatory disorders, and hypotension with dialysis. There are some considerations to examine these drugs in patients with cancer, arthritis, or stroke. Exhaled nitric oxide correlates with the severity of asthma, and plasma and urine levels of nitrite/nitrate have been examined in patients with inflammatory disorders.

Some laboratories have developed a variety of nitric oxide prodrugs or nitric oxide donors to replace currently approved nitrovasodilators. Novel activators of soluble guanylyl cyclase and inhibitors of phosphodiesterase are also in clinical trials.

The studies with nitric oxide have expanded markedly in a number of directions since our original publications in 1977. Today, there are more than 45 000 publications in the field, and there is no indication that it is slowing down.

Not all of the effects of nitric oxide are mediated through soluble guanylyl cyclase activation and cGMP accumulation. Nitric oxide can result in nitration reactions with thiols in proteins to form nitrosothiols. It can also react with superoxide anion to form the very reactive peroxynitrite. Peroxynitrite among other things can nitrate tyrosine residues in proteins. The physiological significance of these reactions continues to be examined.

I expect that the interests in nitric oxide will continue for some period of time as more biological processes are regulated by this simple free radical. I also expect that this field will lead to the development and approval of many novel therapeutic agents for a variety of diseases. It has indeed been very exciting to see our basic research lead to numerous clinical applications. My only regrets are that we did not file any patents in the field in the early years when commercial application became apparent. It could have saved a lot of grant writing today and provided handsome support for the laboratory.

References


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