June 2011 Issue | Nathan Bryan, PhD The University of Texas

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Welcome to *Functional Medicine Update*. What a time this is. We just finished the 20th anniversary meeting of the Institute for Functional Medicine at its annual Symposium in Bellevue, Washington. As I reflect back on my history with the Institute for Functional Medicine, and the concept of functional medicine that we've been defining over these decades, I'm reminded that a very interesting understanding has evolved of how function in the human body results from the interactions between the physiology of a person and his or her environment. It's this gene-environment interaction that leads to specific personalized responses that we call health outcomes.

We cannot modify the structures of our genes directly, but we can modify the environment that we expose our genes to. The story that has been evolving over the last decades is that of the epigenetic modulation of our genetic message. It's been very interesting to follow the evolution of this concept of epigenetics, or modulation "above" the genome of its ability to express its function, in *Functional Medicine Update*. Epigentics encompasses the methylation, acetylation, ubiquinitation, and phosphorylation patterns of the genome and the histone code of the genome (the "book of life") that regulates how the genes are expressed, and how environmental factors and experiences in life, starting even pre-conceptually and moving through early fetal development and into infancy and even (now we're recognizing) adulthood can modulate the epigenetic patterns or the marks on the genome that regulate its function.

This story related to function has really changed its complexion over the last 20 to 30 years, and chronicling this story in *Functional Medicine Update* has been a privilege and an extraordinary "ah-ha" experience for me. It's been like being at the side of a great author who is writing a tremendous novel, and being able to read each page as it comes off her or his pen. It's very interesting to watch the evolution of knowledge.

In this issue of *Functional Medicine Update*, we're going to be talking about a component of this story. During the early days when the tenets of functional medicine were being discussed, we talked about the difference between a differential diagnostic model (the traditional pathophysiologically based model) and

the functional medicine model. We talked about how the diagnosis, in functional medicine, is less important than knowing the etiology of the condition.

Review of the Functional Medicine Model

Antecedents, triggers, mediators, and signs and symptoms are now the sine qua non in functional medicine. Antecedents encompass the genetic background, the family history, and the environmental factors that relate to a particular individual. Triggering events are stimulations in the environment that actually modulate the expression of the antecedent factors. Mediators are the modulators (the messenger molecules) that modulate function at the tissue specific/cellular specific level that ultimately give rise to the outcome that we call function. Outcome is measured in a patient with disturbed metabolism or disturbed function as things such as signs and symptoms of increasing duration, frequency, and intensity. That is the functional medicine model, as contrasted to the differential diagnostic/driving to the disease nomenclature model.

Mediators, these signaling molecules, are like smoke that tells us something about the fire. Often the mediators, in medicine, get relegated to the term “biomarkers.” Biomarkers could be primary or secondary. They could relate specifically to a disease entity (primary), or they could be secondary biomarkers that kind of--using Plato’s Myth of the Cave analogy--give us shadows of understanding as to what the origin of the dysfunction might be. Established biomarkers—clinically validated biomarkers—are things like serum cholesterol or blood pressure. With biomarkers such as these, we use an anatomical, physiological, or biochemical tool to evaluate what’s going on at the cellular level, which reflects how the genes and the environment are interacting in that individual. It’s working backwards towards an understanding of the origin of function or dysfunction.

Inflammation and Disturbances in Metabolism Associated with Aging

I interviewed Dr. Garry Egger from Australia recently, who talked to us about disturbed metabolism and altered function in the world that we live in today (21st century society)--this alarm reaction that the body goes into, this inflammatory response. It could be inflammation of the nervous system associated with Alzheimer’s or Parkinson’s, or it could be inflammation of the vascular endothelium related to heart disease, or it could be inflammation of the beta cells of the endocrine pancreas related to diabetes, or it could be inflammation of the angry fat cells—the adipocytes—that release their adipocytokines that are inflammatory molecules that relate to a variety of things, including non-alcoholic fatty liver disease or non-alcoholic steatohepatitis (NASH). What we’ve said is that inflammation appears to be a marker of disturbances in metabolism that are associated with the dysfunctions of our age.

Dr. Egger talked about how we would examine inflammation at a whole-organism level, and how

environmental factors play a role in that inflammatory process. The example is a recent paper he and his colleagues, David Sullivan and David Colquhoun, published in the *British Journal of Nutrition* in 2010 paper titled "Differences in Post-Prandial Inflammatory Responses to a 'Modern' versus Traditional Meat Diet."[1]

In this preliminary study, Australian individuals who ate Wagyu beef (a feedlot-fed, high fat, marbled, very tender, gourmet beef), and elevated levels of various types of inflammatory mediators (so-called mediators that are biomarkers of disturbed metabolism or altered function) were measured as a consequence of eating that type of diet. If, however, the individuals ate the same amount of wild meat (kangaroo) that was very low in saturated fats and much higher in animal protein on a percent calorie basis than the Wagyu beef, there were not elevations of things like tumor necrosis factor alpha (TNFalpha), and C-reactive protein, and interleukin-6, which are all proinflammatory mediators.

This is a classic example of how an environmental change—a diet of wild game instead of Wagyu beef—produced a very different physiological response across a wide range of different genotypes. This suggests that there might be some people with polygenomic characteristics more highly sensitive to that change, so they might get a very rapid increase in inflammatory markers. Maybe other people, based on their genetic predispositions and sensitivities, would have a lower response. But overall, the findings of this study indicate that when you give different types of meat to a random population in Australia with different genotypes, you get a shift in physiology towards a proinflammatory state.

I think you can line up proinflammation as a characteristic of mediators that are associated with triggers that are associated ultimately with the onset of a variety of chronic age-related diseases: dementia, heart disease, diabetes, maybe certain forms of cancer, certain arthritis relationships to things like osteoporosis, fatty liver disorders, inflammatory bowel disease. All of these can tie into a disturbance of metabolism associated with an environmental perturbant. This has been a general theme I've been developing in *Functional Medicine Update*, and has really been a hallmark of the conceptual framework of functional medicine that differentiates it from that of a histopathology-focused, disease-diagnostic model.

Nitric Oxide: An Important Physiological Mediator Molecule

This month I was very fortunate to interview a leader in this field of cell signaling. In this case the cell signaling substance is nitric oxide. Nitric oxide was the Molecule of the Year. It won the Nobel Prize in medicine and physiology for three extraordinary investigators who independently discovered nitric oxide as being an important physiological mediator molecule. Nitric oxide, or NO, is nitrogen and oxygen (not nitrous oxide, which is laughing gas). NO was found by Ferid Murad and his colleagues, and by Farragut in a different lab, and was ultimately recognized to be a very important modulator of physiological function related to signaling through the guanosine monophosphate signaling pathway (or cyclic GMP

pathway), that has to do ultimately with phosphodiesterase, endothelial function, and vascular tone.

Sildenafil: A Drug Designed for Hypertension Becomes a Lifestyle Drug and Nitric Oxide Gains Commercial Interest

Later, this got translated into an interesting commercial application through a drug that was being explored as an anti-hypertensive drug that had effects on nitric oxide, cyclic GMP, and phosphodiasterase. It was a drug called sildenafil. In the studies that it was being employed in to evaluate its effect as an antihypertensive, sildenafil had an off-target effect in males: it increased erections. I can imagine the marketing staff at Pfizer said, “Now hold on. We’ve got two applications for this drug. One is the indication to treat hypertension, and there are many medications in that class. And then here’s another interesting…not disease application, but certainly what we might call a lifestyle drug application for treating a condition that we can promote to become a really major new syndrome: erectile dysfunction syndrome (ED). We’re going to promote this as being a pandemic problem, and here’s a solution to it.”

Sildenafil ultimately became a billion-dollar, blockbuster addition to a category now called lifestyle drugs. That medication is basically managing this defect in nitric oxide production by the vascular endothelium. That really took the nitric oxide story to a new level of interest commercially, and built a huge business around it.

But leaving behind the erectile dysfunction component, there is this whole other story about endothelial tissues that line the whole surface of our body and how they relate to environmental agents and ultimately trigger the release of mediator substances that regulate the tone and function of those tissues. It’s that story that we’re going to hear much more about in this extraordinary interview with Dr. Nathan Bryan, who is the author of a well-written and highly information-rich book (a very readable book) titled *The Nitric Oxide Solution: How to Boost the Body’s Miracle Molecule to Prevent and Reverse Chronic Disease*.[2]

INTERVIEW TRANSCRIPT

Researcher of the Month

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Once again we're at the high point of the edition, which is our Clinician/Researcher of the Month. Nitric oxide was the molecule of the year about 15 years ago. I've talked about the nitric oxide story and its interrelationship to physiology in Functional Medicine Update before. The three forms of nitric oxide synthase are the endothelial, the neuronal, and the inducible immune. I've had the pleasure and privilege of talking with two Nobel Prize winners in medicine and physiology who were in the group of three that discovered this nitric oxide physiology connection: Dr. Ferid Murad (right after he won the Lasker Award for his work) and also Dr. Louis Ignarro from UCLA, one of the co-recipients of the Nobel Prize. Today, there is even more clarity and understanding of the nitric oxide physiology story. I'm excited to talk with a person who is working with Dr. Murad in this field of nitric oxide physiology, Dr. Nathan Bryan.

Let me tell you a little bit about Dr. Bryan in case you are unfamiliar with his name. He is the author of a book on this topic: The Nitric Oxide Solution: How to Boost the Body's Miracle Molecule in Preventing and Curing Chronic Disease. It is a very descriptive overview of the extraordinary research work that Dr. Bryan and his colleagues have been doing at the University of Texas (UT) Health Sciences Center in Houston. Beyond that, he has been extraordinarily involved in the fundamental research in this area as faculty working in the school of biomedical sciences at the UT Houston Medical School.

Dr. Bryan is an active member of the Nitric Oxide Society. He has been published extensively in Free Radical Medicine and Biology, and he is working with the American Heart Association as a member of the AHA. Dr. Bryan was recognized as the university's most accomplished young investigator in 2007 (not a small achievement whatsoever). He has published over 30 peer-reviewed papers in top-flight journals, and he has been cited over 1200 times for the pioneering work that he is doing in this area, which ties together with nutrigenomics and the gene-environment connection that we have focused on so heavily in Functional Medicine Update.

Dr. Bryan, with great thanks we welcome you to Functional Medicine Update. Maybe you can help us get into your fascinating story by giving us a little bit of background about what led you to this work on this extraordinary molecule?

NB: Thanks, Dr. Bland, for the great introduction. It's certainly a privilege and honor to join you today. We're certainly excited about where the field is moving. We think we've made some seminal discoveries that really are changing the paradigm of how we look at nitric oxide regulation and production within the human body.

I've been in this business for over 10 years now. I started out training under Martin Feelisch, a pharmacologist who was involved in the early work on nitric oxide, looking at the mechanism of action of nitrovasodilators. After that I was at Boston University School of Medicine and worked under Joe Loscalzo in the Department of Medicine at the Whitaker Cardiovascular Institute. And then I was recruited by Ferid Murad at the Institute of Molecular Medicine in Houston to join their drug discovery program in trying to figure out how to develop and create novel, safe, and effective nitric oxide-based therapeutics. Thirty years after the discovery of nitric oxide (or what was then endothelium-derived relaxing factor), and 12 to 13 years after the Nobel Prize, there has really been no hallmark discoveries in terms of novel therapeutics aimed at restoring nitric oxide physiology or homeostasis.

That's where we've come in and—I think—made some important discoveries. Being trained as a physiologist, I was always taught that the body has an enormous redundancy, so there is more than one pathway to generate such a fundamental molecule. Today, really the only pathway discovered or described in the literature is through the 5-electron oxidation of l-arginine, so this l-arginine-nitric oxide pathway. It's inside probably one of the most complex and complicated reactions that takes place in the human body. Why would nature divide such a complicated and complex pathway to create such—what we think—is one of the most important and fundamental molecules in physiology?

Nitrite Has Been Found to Be Bioactive and Cardioprotective

Serendipity really factored into where we are today. We were looking at how people have used the oxidative end products nitrite and nitrate for 20-plus years as kind of proxies or biomarkers for local nitric oxide production. What we found back in the early 2000s was that these molecules, particularly the nitrite anion, have some biological activity. We published a paper in Nature Chemical Biology showing that nitrite can actually modulate post-translational modification of proteins through these nitrosylation events that can induce gene expression and elicit cell signaling events.[3] So it wasn't this innocuous, inert biomarker of nitric oxide; in fact, it was actually bioactive itself, particularly under conditions where the NOS (or nitric oxide synthase) production of nitric oxide becomes dysfunctional.

We, and others, have done a lot of studies since looking specifically at the physiological activity of nitrite, and what we're finding is that it is extremely cardioprotective. If we subject, in experimental models, heart attack or stroke and have this anion on board, we see enormous protection from injury in the brain from stroke, and enormous protection in the heart from a heart attack. We study finding ways that we can restore nitric oxide homeostasis independent of this nitric oxide synthase production of nitric oxide.

JB: I have had the chance to read your really fascinating 2005 paper in Nature Chemical Biology that talks about nitrate as a signaling molecule and a regulator of gene expression. I think this is a very interesting new chapter you are describing. I know many people have historically looked at urinary nitrate as a surrogate marker for nitric oxide. Given what you said, is there some ratio between urinary nitrite versus nitrate that is more specific in terms of assessing nitric oxide physiology, or is it looking at the total of the sum of nitrite plus nitrate in the urine that kind of gives you a surrogate evaluation?

Measuring Specific Nitric Oxide Biochemistry is Proving Complicated

NB: If it were only that simple. In assessing biological samples from patients in the clinic, sampling a single compartment at a single time point really gives you very little information. What we've always tried to do is take plasma samples, urine samples, or any tissue or biological sample we could from patients at the same time and over time, because really the renal excretion of these two anions depends a lot on renal function, so people with renal insufficiency or renal failure are going to have different excretion profiles from otherwise healthy people. What's happening in the urine is completely different from what's happening in the plasma, and we know from experimental animal models that what's happening in the plasma isn't always a direct reflection of what's happening locally in a specific tissue of interest (in the heart, for example). In terms of fingerprinting specific nitric oxide biochemistry in a specific tissue bed, it's really difficult to get a handle by measuring either urine or plasma samples, but we're developing techniques and technologies that give us a better handle on what's going on. Typically nitrate is the oxidative end product, and there is some reabsorption that happens in the proximal gut which

then mixes with the nitrate in your diet and what's produced endogenously through the oxidation of nitric oxide, and then there is some tubular reabsorption in the kidneys of this anion and then what's left over spills out into the urine. Broadly speaking, you can get a feel for total body nitric oxide availability by measuring certain biological compartments over a period of time.

JB: I know you've done some work on looking at salivary nitrite as a surrogate secondary biomarker. Does this appear to have some broad brush potential in evaluating aspects of nitric oxide status?

NB: It does. We're actually very excited about this. One of the things we've been trying to do as we move this field forward is to bring more awareness about nitric oxide. We, in the biomedical science arena, are really excited about it and we've known about it for 20-plus years, but if you go out on the streets and ask the lay people, "What is nitric oxide?" I'm guessing probably 75 to 80% of people don't know what it is or they confuse it with nitrous oxide, the anesthetic they get when they go to the dentist. We realize that there is a huge educational hurdle to overcome. You can't go to your physician and ask for a lab to determine your nitric oxide level like you can for vitamin D, or get a reading for your cholesterol, or for magnesium, or any other blood labs that are standard measurements.

Knowing that, we've been doing correlation studies for a number of years and trying to get a sense of a way that we can noninvasively determine a person's nitric oxide status. As we mentioned, there is an entero-salivary circulation (a recirculation) of nitrate/nitrite and that then creates what we call a human nitrogen cycle. For instance, about 25% of the nitrate that is produced within your body and also what you take in through eating certain foods (for instance green leafy vegetables) is absorbed in the proximal gut, and then that's concentrated in your salivary glands, so there is an entero-salivary circulation.

Colormetric Evaluation of Salivary Nitrite May Be A Powerful Screening Tool

Each time you salivate you get a burst of nitrate in your mouth. And then the cyclic anaerobic bacteria that reside on your teeth and in the crypts of your tongue then have a functional nitrate reductase. This nitrate reductase produces salivary nitrite levels that are anywhere from 100 to 1000 times higher than the concentration that is found in your plasma. Each time you swallow you get a burst of nitric oxide because the PKA of nitrite is about 3.2, so in the acid environment of the stomach each time you swallow, provided you have sufficient entero-salivary circulation, you get a burst of nitric oxide in the stomach. This pathway has been shown to enhance gastric mucosal blood flow, increase absorption of nutrients, and prevent overgrowth of food borne toxins or bacteria, including H. pylori. So it's really an essential pathway for modulating nitric oxide homeostasis. What we realized then was that we could capture this through a colorimetric noninvasive diagnostic. We developed really the first and only nitric oxide diagnostic through the UT Health Science Center, Houston.

JB: I've seen this and I think it is really fascinating. It's basically a dipstick that does colorimetric evaluation of saliva nitrate, which to me is a very, very powerful kind of screening tool. Have you had some occasion to clinically evaluate how this matches nitric oxide status?

NB: We have, and we took really an extreme population. We took people that have been on chronic dialysis, so people with kidney failure, because really these people suffer from usually a 10 to 20 times higher incidence of cardiovascular disease. These people die of cardiovascular-related events, not anything to do with their kidney failure (although one could argue there's a direct connection).

We recently just completed a study where we sampled the blood from these dialysis patients going into the dialysis unit and coming out of the dialysis unit. We sampled their blood and saliva after 4 to 5 hours of dialysis. What we saw and found was the salivary levels of their nitrite and nitrate were actually much more predictive of the scavenging effects of this chronic dialysis procedure in scavenging nitric oxide than what was reflected in the blood (or I should say just as predictive). We're finding that if you increase endogenous nitric oxide production either through therapeutics such as organic nitrates, or if you increase endogenous nitric oxide production by eating certain foods or adopting a diet that may be rich in nitric oxide activity, then you can enhance this nitric oxide production through this entero-salivary circulation. And if you become nitric oxide deficient or depleted, then we can see a reduction in the entero-salivary circulation or the recirculation of nitrites.

Obviously this is new technology and we'll do more validation studies as time goes on, but as we stand right now we have a pretty good correlation between total body nitric oxide production or availability in what we are able to pick up through our salivary test.

JB: That's an exciting next step in kind of making this more real to the clinician, having the availability of a surrogate marker. Let's now talk about nitric oxide at the physiologic level. When we first learned about this we learned about the three isoforms of nitric oxide synthase or NOS, which were the neuronal, endothelial, and immune-inducible, and we had this concept that two of the forms were constitutive and one of the forms was inducible. I think since then much has traveled to better understand the form of these enzymes that are involved with nitric oxide production and how they relate to physiology. Could you give us an update on that as it relates to what's now known?

NB: Right. That was obviously a misnomer, as we know now and as you pointed out, because there are constitutive forms of the inducible isoform (you can actually induce or up-regulate the constitutive eNOS and nNOS), so now they are commonly referred to as isoforms NOS 1, 2, and 3. The constitutive NOS is still recognized as a calcium-dependent, tightly-regulated production of nitric oxide where with the inducible (the inflammatory) component you get this chronic, nauseous overexposure/overproduction of nitric oxide. What we have most recently realized is that you can specifically modulate the constitutive (or what we think are the beneficial) isoforms of nitric oxide, specifically through the modulation of citrulline in the body, so via the urea cycle in our body. L-arginine is classified as a semi-essential amino acid, meaning that your body produces it but not in a net effect that it is sufficient to sustain all metabolic activity, so you need L-arginine from the diet. But what we are realizing is this urea cycle production of L-arginine, specifically from L-citrulline, is this particular pathway preferentially shuttles L-arginine into this constitutive nitric oxide pathway.

The L-Arginine Paradox

As you are probably aware, there is an L-arginine paradox, and the paradox lies in the fact that the Michaelis constant (Km) and the saturation kinetics for the NOS enzyme is about 5 micromolar, but intracellular concentrations of L-arginine are typically 100 to 200 micromolars. Even under basal steady state conditions, the NOS enzyme is theoretically saturated, so the paradox lies in the fact that if you give L-arginine, in some cases you can then produce more nitric oxide. There are a number of hypotheses out there on how to do that, though. Lou Ignarro suggests—and I think it is probably the prevailing paradigm—that you may be out-competing the endogenous inhibitor, asymmetric dimethylarginine. But we are finding is that you can actually preferentially shuttle L-arginine into the nitric oxide pathway by

giving the body citrulline. You are basically providing the body with the substrate it needs to locally and specifically turn that citrulline into L-arginine that can then make nitric oxide.

There are at least eight metabolic pathways to utilize L-arginine, and it is estimated only about 5% goes to the nitric oxide pathway, but we can make this much more efficient through using L-citrulline instead of L-arginine.

JB: This is fascinating, I think, on multiple levels. For those that are not biochemists that are listening—a preponderance of our supporters are clinicians who may not be completely up on their enzyme kinetics and enzyme activity—let me just say a couple of quick words about what we just heard from Dr. Bryan.

This concept that there is this difference between the Km value of the enzyme and the in situ concentration of its substrate, suggesting that at the concentration in situ that you are completely saturating the enzyme, so giving more wouldn't necessarily have any more beneficial effect because you are already totally loading it. But yet clinically you see effects, even against the paradox of this enzyme kinetic saturation. It is not only very interesting relative to arginine and its relationship to nitric oxide, but I think there is a general concept that may be very interesting as it relates to the role of a number of putative precursors to various substances that are regulators of intercellular signaling and have some nutritional relationship.

For years we've had this discussion about mass action effects, and actually it comes out of Linus Pauling's article in 1967 in Science magazine titled "Orthomolecular Psychiatry," where he talks about Km and talks about concentrations and pushing, by Le Chatelier principle, equilibrium more to the right.[4]

Some of these pathways are more than just linear pathways, as Dr. Bryan is pointing out. There can be circuitous routes in order to facultative respond to metabolic pressure by increasing substrate concentrations that are not just this linear A-goes-to-B type of model. I think this is a very important chapter, in general, to our understanding as to how substances may work at higher concentrations than would be normal. We say, "But the enzyme, for its single pathway, is already saturated. There may be multiple routes to conversion." That's number one. Number two, which I would like to ask you to follow up on is when nitric oxide is produced through maybe this interrelationship of the citrulline-arginine conversion pathways and these eight different metabolic pathways, is it then serving as an autocrine, a paracrine, or an endocrine messenger, or is it all of them?

NB: We have evidence to believe it is all. Great point on the follow-up for the enzyme connection. I think you're spot on in terms of it is not just a single pathway, it's much more complicated than that. When nitric oxide is produced, it's a gas so it is produced by endothelial cells, and its first function is that it diffuses into the underlying smooth muscle, so it acts on a neighboring cell in a paracrine fashion. Once nitric oxide is produced it only stays around for about a second, and much less under certain conditions. The question has always been: Is there an endocrine function of nitric oxide? It is so short-lived, how can it survive transport in a sea of oxyhemoglobin, which is its known scavenger, to elicit distal functions?

We've played with that question for a number of years, and in 2007 we actually demonstrated this—quite convincingly, I think—through a paper we published in the Proceedings of the National Academy of Science with Dave Lefer's group, who was then at Emory.[5] We over-expressed the nitric oxide

synthase enzyme, specifically in the heart. If you subject these animals to left anterior descending artery ligation and subject them to a heart attack, they are very protective. They heart sees very little injury. They recover quite nicely, and they do very well.

What we also did in these mice, even though nitric oxide is over-expressed and over-produced, specifically in the heart when we measured their liver and blood levels of nitric oxide, they were elevated as well. And then if we subjected these animals to hepatic ischemia reperfusion injury, we found that there was enormous protection from injury for IR injury. Obviously there was an endocrine function because the nitric oxide that was only over-expressed in the heart then spills out into the circulatory system. It is transported into distal tissues and elicits a level of protection from insult. We recognize it is one of two (if not both) carrier molecules. One was nitrite, which was elevated, which we know is tissue protective, especially under ischemia reperfusion conditions, and also nitrosothiol, so these low molecular weight nitrosothiols that can then be transported through the blood and elicit these trans-nitrosations that may affect protein structure and function.

JB: This is why these conversations are so exciting, because you never know how they might morph and where they might take us. First of all, I just want to compliment you on that PNAS paper, “Dietary Nitrate Supplementation: Protecting Against Myocardial Ischemic Reperfusion Injury.” I think it’s a brilliant bit of work—a really well-written, very articulate paper. I think the companion paper, is also from your group, in the Journal of Biological Chemistry in 2008, “Tissue Processing and Nitrate in Hypoxia.”[6] It seems to frame a whole different model, or let’s say the next step in our understanding of this nitric oxide/nitrate/nitrite connection.

Now let me, if I can, ask you the following clinical questions. A lot of our listeners might be saying, “Oh boy, I’ve been administering arginine to patients to try to enhance their nitric oxide production, but given what I’ve just heard you say, is it possible that I could be enhancing aspects of nitric oxide that might be deleterious to as patient that has inflammation onboard because I’ve now maybe done something or upset an important equilibrium in a harmful way?” What’s our thought about that question?

Clinicians Should Consider Adverse Events Related to L-Arginine Therapy

NB: I think that’s a very good point. I’ve seen anecdotal evidence in the clinic of people having adverse events. In fact, there was a JAMA paper published in 2006 where they tried to give post-infarct patients L-arginine therapy after a heart attack to see if they could improve patient outcome from the heart attack. They had to stop the trial because the L-arginine was actually killing more people than the placebo.[7] I think without understanding the context of that little black box from arginine to nitric oxide, that naiveté is what leads to these adverse events, I think. As I mentioned, it’s a 5-electron oxidation requiring six different co-factors and substrates, and if one or several of those substrates aren’t at the right place at the right time, you can actually get superoxide produced instead of nitric oxide, thereby exacerbating any condition.

There are two things there: 1) not understanding the full context (if you give L-arginine with some antioxidants to keep this reduced co-factor pool reduced and available to be used by the nitric oxide synthase enzyme, then it becomes much more effective); 2) the underlying inflammatory condition (if you just seed this enzyme L-arginine, the inducible isoform utilizes L-arginine too, so you may be fueling that inducible isoform by feeding L-arginine and really not feeding the constitutive form which is what

you really want to be doing.

So there is enormous complexity in the regulation, and specifically modulating the constitutive (or beneficial) isoforms of nitric oxide. How you segmented this is trying to understand: How do we restore nitric oxide homeostasis in a patient population? I think part of it is through this L-arginine oxidation pathway, but I think at least half of it (if not more) comes from a dietary influence. We've done a number of studies looking at nitrogen oxides that are found naturally in foods (particularly green leafy vegetables) that can then undergo this reductive pathway from nitrate to nitrite to nitric oxide. This seems to account for—in our hands and many others—about 50% of the bioactive pool of nitric oxide. This was exciting for us because then we had a dietary intervention to overcome so-called endothelial dysfunction, or an insufficiency of endogenous nitric oxide production.

Seeing Nitrites in a Different Context

JB: Which translates clinically into hypertension and poor vascular endothelial responsiveness. I think the article that you were the principal author of that appeared in the American Journal of Clinical Nutrition in 2009 titled "Food Sources of Nitrates and Nitrites: The Physiological Context for Potential Health Benefits," is really a landmark paper because in many peoples' minds nitrites are only associated with methemoglobinemia, and they're a problem, and they should be removed from the diet, but this puts a whole different context and spin on it.[8] Have you had people raising their eyebrows at all when you talk about the beneficial effects of nitrates and nitrites in the diet?

NB: We have. We get a broad spectrum of response, sometimes a visceral reaction from people who grew up in the 60s and 70s who were taught that these were human carcinogens, they were unwanted contaminants in our food and water supply. But you know that was long before it was ever discovered that these anions were actually produced within our bodies, so they're not contaminant foreign food additives; they're actually naturally occurring molecules that are produced within our bodies. The notion that they're toxic…you know, nothing is without toxicity. Obviously there is a context for health benefits and for toxicity and it has to do with concentrations or total exposure. We've gotten mostly positive response because I think this completely changes the paradigm of how we think about nitrogen oxides in our food supply, how we think about modulation of endogenous nitric oxide production, and really then begin to put together a program or a strategy to treat people who may be nitric oxide insufficient. I think it's a combination of fueling that endogenous pathway and creating a reservoir of dietary interventions of a bioactive pool of nitric oxide.

Summary of Nitric Oxide Points

JB: Let me just summarize as a weigh station, here, as we move on to the next series of questions. We've learned a tremendous number of things already from you. We've learned that dietary nitrate and nitrite has an impact upon nitric oxide function and also works directly through some of this regulatory processing to regulate gene expression. Secondly we found that nitrite and nitric oxide both are cardioprotective as well as vascular endothelial active and help in smooth muscle relaxation (vasodilation). We've heard from you that if you use the appropriate kind of nutrient mixture which includes things like citrulline, and nitrite, and various antioxidants, that you can modulate various pathways that are associated with nitric oxide production, not just the single-A-going-to-B nitric oxide synthase pathway. You've also told us now that the role of nitric oxide is far greater than we thought

originally as it relates to its signaling effects. It's more than just its first name: vascular endothelial relaxing factor. Now we're talking about its role in modulating things like bioactive sulfhydryls, and oxyhemoglobin, and many other molecules by nitrosation, which then can have a pleiotropic series of effects, and then that raises, obviously, a question. I think the highest concentration of active thiols intracellularly is in glutathione, which has that cysteine residue (the central amino acid in glutathione) sitting there. What do we know about the interconnection with redox in glutathione and nitric oxide? It seems like that's an emerging part of the story.

NB: It is and I think probably one of the main vascular carriers of nitric oxide activity is through low molecular weight thiols such as free cysteine or glutathione. In fact, it's glutathione or nitrosyl glutathione that acts as the intermediate between these transnitrosation reactions that act to post-translationally modify protein thiols. We always think about it in terms of phosphorylation, so you'd have kinases and phosphorylations that add and remove phosphate groups to proteins that then affect structure and function. We have this same paradigm now with nitric oxide. There are nitric oxide congeners such as either hydroxyl or ML+ that can then directly nitrosate files, either chemically (and there are some enzymatic pathways that have been discovered where you can then modify a critical cysteine residue within a protein and then do a denitrosation).hat was kind of the first new paradigm, particularly put forth by Jonathan Stanley (then at Duke) on a novel signaling aspect of nitric oxide. Then the question was: If this is a signaling event then doesn't there have to be an on/off reaction, particularly an enzymatic denitrosation? There have been a number of enzyme systems now to hide the dehydrogenase system that can denitrosate protein nitrosylthiols through the intermediary of glutathione. As you alluded to, this free file pool, particularly within glutathione, is very, very important and probably critical in modulating nitric oxide modulation of protein thiols.

JB: Now let's take that to a clinical perspective for many of our listeners who are bearing with you and I having a wonderful exotic discussion about biochemistry and are waiting patiently for when this has a clinical application. Let's talk about the patient who is insulin resistant and hyperinsulinemic. A person who has elevated uric acid and/or elevated homocysteine and elevated hsCRP, so they've got an inflammatory biomarker, they've got some indication of alteration in homocysteine/folate processing, and they've got this xanthine/oxidase thing that is seen as an elevated uric acid. And, if they even do more extensive laboratory testing (let's say that they measure asymmetric dimethlyarginine (ADMA) —this would be more of an esoteric analyte but it can be done so they have an ADMA level that is elevated), how would that all fit together with the model that you are emerging around nitric oxide, or what kind of clinical thoughts might derive out of that kind of a patient?

NB: I think it presents the perfect scenario of global nitric oxide insufficiency because everything you talk about there, from a clinical standpoint, talks about dysregulated nitric oxide production. Starting with the first part—the insulin resistance—part of the insulin signaling pathways is this production of insulin by the beta cells, and then activation of certain cell signaling pathways that eventually lead to GLUT-4 translocation and glucose uptake in the cell. Intermediate, in that pathway, is nitric oxide. What we're finding is that particularly in pre-diabetics or even diabetics, they're nitric oxide insufficient or they become dysregulated and that's the reason for the cardiovascular complications or higher incidence of cardiovascular disease in diabetics and why it's a risk factor. If you have a roadblock at that production of nitric oxide then you can't have part of this GLUT-4 translocation pathway as nitric oxide-dependent. What we think is that nitric oxide produced through the insulin signaling pathway leads to a nitrosation event of GLUT-4, which then signals it to translocate to the membrane, take up glucose, and then you get

normal glucose metabolism and uptake. But when you have dysregulated nitric oxide production that event doesn't occur. Although you're getting sufficient insulin production, you're not completing the pathway. We think if you can restore normal nitric oxide signaling or homeostasis then you can eliminate that roadblock and complete the insulin signaling pathway. The inflammatory biomarkers of the CRP—obviously I think a lot of people (biochemists and physiologists) consider nitric oxide an anti-inflammatory molecule because it can actually squash the inflammatory response. And then the uric acid—the xanthine oxidase--is one of the prime mediators, or it's an active nitrite reductase, so you can actually out compete that uric acid production pathway by having sufficient nitrite around to where we can have another source of nitric oxide. Actually we have seen, anecdotally, people that we've given our nitric oxide intervention to that have less flare-ups of gout and actually see symptomatic relief.

Clinical Connections to Nitric Oxide

JB: Thank you. I hope you clinicians now feel a sense of satisfaction—like taking a deep sigh, here—that now you've endured our biochemistry to recognize that that patient with the marginally elevated uric acid, and maybe homocysteine elevations, high sensitivity CRP, and some insulin resistance with central adiposity, and if you measured ADMA in their blood, you might find it elevated: there you go. They might have marginally elevated blood pressure too, and they might have increased carotid intimal medial thickness (or CMIT tests are not looking so good). Think nitric oxide, and think the citrulline/arginine/antioxidant/nitrite connection. In fact, Dr. Bryant authored a very interesting paper that really talks to this titled "Dietary Nitrite: Preventing Hypercholesterolemic Microvascular Inflammation in Reversing Endothelial Dysfunction."[9] This was in the American Journal of Physiological Heart Circulatory Physiology in 2009. There's lots of stuff to support what we're alluding to here.

Let me ask the next question that follows on the heels of this. Because you've talked about dietary nitrates and nitrites playing a role in the systemic production of nitric oxide and the role that nitrite itself has, that also begs a companion question and that is: Are there known phytochemicals that could modulate this pathway not through direct nitrite contribution, but through effects through kinase pathways or other regulatory mechanisms to influence the enzymes that are then are involved with nitric oxide production? I know the answer to that question because you authored a paper on this looking at traditional Chinese medicines and the relationship to nitric oxide bioavailability. What about phytochemicals and the role of nitric oxide production?

Nitric Oxide and Traditional Chinese Medicine

NB: Yes, I think they're absolutely essential. I can't take full credit for that. I had a post doc contact me several years ago who was trained in traditional medicines; he was an MD from China. He was working here in the US as an interventional cardiologist and he was interested in some of the work we were doing and he said, "I wonder if there is a connection between the traditional medicines we use in China to treat cardiovascular-related diseases and nitric oxide production?" We actually just did a very simple experiment and got really profound results. He went to a local acupuncture shop and I told him to purchase only herbs or botanicals that were used specifically for cardiovascular indications. We put them through our nitric acid assay. We looked at how they activated soluble guanylate cyclase, how they activated endothelial nitric oxide production, as well as their ability to turn these anions, nitrate and nitrite, into nitric oxide. What we were thinking was kind of a rescue pathway to restore nitric oxide, because most of these herbs contain very, very high concentrations of nitrate, and in some cases nitrite.

To kind of preface the importance of that, and really the importance of the phytochemicals and this whole pathway, is the fact that we've done a number of studies—particularly the JBC study in 2009.[10] Mammalian systems are grossly inefficient at converting these anions back into nitric oxide. There is a three-leg order of magnitude of inefficiency from nitrate to nitrite to nitric oxide. One could argue that from the physiological concentrations that we have in our blood and our tissue, can you get any appreciable amount of bioactive nitric oxide from the steady state concentrations that are normally there? One can argue that, but people who have endothelial dysfunction and risk factors for cardiovascular disease, clinically they have less nitrite and nitrates in their blood and their tissue. So then, when they need that pathway even more than healthy people, we're so inefficient that we can't do it. My point is that we've recognized traditional medicines are herbs and phytochemicals that do this reaction for us. When you ingest these in traditional medicines, they provide this reductive pathway to then take the inorganic nitrate to nitrite and the nitrite, particularly, to nitric oxide. This then becomes an oxygen-independent reaction that's very, very efficient. You can actually pick up this activity in the blood after you've taken these medicines. Whatever it is, it's surviving first-pass metabolism.

When we got really excited about that, we were interested in trying to isolate and identify that active component that was responsible for this. We did a number of extractions and counter current chromatography to try to identify the active components to where we could then synthesize and call it a drug, which was the business we were in at the time. Not surprising to naturopaths and people who use functional medicine, when we fractionated the parent compound, the activity fell apart. So it was really the synergistic effects of the parent compound in its unrefined form that had the activity and it wasn't any one particular component. In terms of drug discovery we really hit a roadblock and we couldn't move forward. We scent screened probably over 200 traditional medicines and botanicals and found really a handful of hits that are very, very effective at this reductive recycling to nitric oxide. We realized that if we could harness that activity and its parent compound form, then we would probably have something better than a drug—something that didn't inhibit a single pathway but acted synergistically that restored nitric oxide production. You could harness that activity, and hopefully without all the adverse side effects that many drugs have.

JB: I want to really compliment you. This paper that you authored with your post doc in Free Radical Biology and Medicine in 2009 is titled "Nitric Oxide: Bioavailability of Traditional Chinese Medicines Used for Cardiovascular Indications" and is a really interesting and provocative step forward in our understanding of how agents within traditional medicines influence these regulatory pathways.[11] I think in the article you write about frankincense and red peony root and ginseng as part of the several hundred things that you studied having effect. I know you've seen things with horse chestnut extract as well. I think this is a very powerful contribution to our understanding of this whole field. It is more than just the amino acid, arginine, or the amino acid, citrulline. There is a whole variety of different things in our environment that influence this regulatory pathway.

NB: Right. Yes, we're excited about it. We think it now creates a new strategy to intervene naturally and restore normal nitric oxide production.

Nitrate and Nitrite Measurements in Breast Milk, Bovine and Soy Milks

JB: Let me close with one last part of this story. This is certainly not the end of this story; we could spend hours talking with you. I know we've just touched the surface of the envelope, here, of the things that

you are working on and the things that you've discovered. One thing that I think our listeners would find very interesting is this whole concept of infants. They are born with a sterile gut and obviously their conversion from microbes may be different from that of adults that have gut resident colonization, and they've got biofilms, and a microbiome that is working as a secondary conversion factor for them. You've authored this recent paper in Breastfeeding Medicine talking about "Nitrate and Nitrite Content in Human Formula, Bovine and Soy Milks: An Implication for Vascular Health in Infants."[12] Maybe you could tell us a little bit more about this. It's a very interesting chapter in the story.

NB: Yes, this is interesting. I'm curious by nature. Three years ago my wife gave birth to our now three-year old. Kind of nonchalantly, the nurses and the physician, when we left the hospital, said, "You'll want to breast feed [and this was our second son], but if you don't then give the baby this formula." I got to thinking about what's in this formula that is not in the breast milk and vice versa. After a little bit of a struggle, I convinced my wife to go to my lab and I expressed some of her breast milk (at the time, colostrum) and then did a biochemical comparison to what was in the formula. What we found was astounding: there was about 25 to 30 times more nitrite and nitrate in that early colostrum—in that breast milk—than what was in that commercially available formula they gave us when we left the hospital.

I wanted to know if it was just an anomaly (that she, being my wife, probably had high nitric oxide activity in her breast milk). We got an IRB approved. We tested over 70 mothers that were admitted to Memorial Herman Labor and Delivery, and we sampled breast milk throughout postpartum time periods, from day 2 all the way up to 4 to 5 weeks postpartum. What we found was the ratio of these anions changed with the transition of milk. As you are probably aware, the early milk is the colostrum. People think it is high in immunoglobulins. It is really the essential nutrients for these first days of life. After day 4 to 5 to two weeks, you get what you call transition milk, and then from two weeks on it is what's classified as mature milk.

Physiologically, what we think is happening is when the infant is born, they have a sterile gut; there is no bacteria that are colonized in their gut. We as humans don't have a functional nitrate reductase; we rely on the bacteria in our mouth and in our gut to perform this. As the gut becomes colonized over the period of the first several days, nature has provided a way—we think—as a source of nitric oxide. It's the nitrite in the milk that when the baby takes the colostrum it's reduced to nitric oxide in the stomach and the gut, but then as the bacteria begin to colonize, there is really a perfect overlap in the time of colonization to the timing of the change in these anions taking place. Once the bacteria are colonized, then they have the machinery in place to reduce the nitrate in the milk to nitrite, which then becomes nitric oxide.

Clinical Importance of the Breast Milk Study

This becomes really exciting and important clinically in, particularly, a condition called necrotizing enterocolitis (NEC). Premature babies that are subjected to hypoxia and are in the NICU are fed formulas developed for this and I think it's still about a 20% mortality rate. Babies that are breast fed don't develop NEC. We have some very preliminary data showing (and we have nanomodels of this): if we replete the missing nitrite in the breast milk and just fortify it in the formula, we can completely prevent the development of necrotizing enterocolitis. In fact, we're about to take this into the clinic. From a neonatal standpoint, this nitric oxide story stays with us from the time of birth until we pass on, so it is something that we can't ignore at any point in life.

JB: First of all, the last sentence in your abstract of that paper I think is very powerful for those of us that think in kind of web-like physiology. I'm quoting: "These data support the hypothesis that the high concentrations of breast milk nitrite and nitrate are evidence for a physiological requirement to support gastrointestinal and immune homeostasis in the neonate." That has a spreading effect of impact other than just the neonate about the whole relationship between gut, immune, and regulatory control mechanisms. I want to applaud that sentence. I think it's a great one.

NB: Thank you.

JB: I also found it very interesting, when you look at your data, and you contrast the nitrate/nitrite levels in colostrum, and transition, and mature breast milk. Let's take mature just as an example. The formulas were extraordinarily low, in general, in these constituents compared to mature breast milk, but some of the soy milks were in the same range as breast milk, which I found interesting.

NB: Right. We've started a study on soy-based products versus their non-soy counterparts (soy milk, regular milk, soy-based proteins versus meat-based proteins). It's a common theme. The nitrate/nitrite content, or what we think is this nitric oxide activity, is much higher in these soy products and I think may be the reason for a lot of the health benefits of soy versus non-soy products or food-based products.

JB: Lastly, Dr. Bryan, for anyone who wants to follow up there is an excellent review article that you have co-authored with Dr. Murad on this whole concept of nitric oxide signaling pathways and targets—a Frontiers in Biosciences article that I think is a very, very good review paper on eNOS, iNOS, nNOS, nitrovasal dilation, the cyclic GMP relationship, how that relates to the Viagra story and sildenafil, which then has to do with vascular perfusion and endothelial function.[13] I just think your contribution to this field and tying it to nutrition and phytochemistry is truly groundbreaking. I would recommend, again, for our listeners, if you want to follow up and get a more simplified explanation of this whole discussion, look at Dr. Bryan's book The Nitric Oxide Solution, which I think you'll find really is news to use.

Nathan, thanks so much for spending this time with us. I know we've taken you away from the laboratory and your work, but we greatly appreciate it. I know our listeners will value from the clinical news to use that comes out of this.

NB: Thank you very much for the invite. I've enjoyed the conversation.

JB: Thank you. The best to you and keep up the great work.

I hope that your appreciation of what Nathan Bryan had to say is as great as mine. That was a very eloquent and informative discussion about this complex topic of nitric oxide physiology, and nitric oxide chemistry, and nitric oxide's role as an intercellular and extracellular mediator, an autocrine- and endocrine-like mediator molecule. Really, really fascinating news to use.

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