Our laboratory studies nitric oxide (NO) biosynthesis in mammals and some of the related consequences of NO release. Discovered in 1987, this novel biochemical pathway is now considered to be a widespread means for host defense, regulation of cell function, and communication. Specific systems in which the pathway participates are signal transduction in the brain, stroke, control of blood pressure and heart rate, gastric motility, and immunologic destruction of tumor cells and microbes. Our main interests are in uncovering the enzyme reaction mechanism, understanding how enzyme structure relates to its function, learning how other cellular proteins can control NO synthase (NOS) activity, and the cell biology of NO as it relates to protein processing and modifications.

Our lab works with three distinct mammalian NOS: A cytokine-induced NOS and two constitutively expressed NOS from neurons and endothelium. We also work with bacterial NOS-like enzymes from Bacillus and other gram+ bacteria. We typically work with purified proteins and enzymes, whose cDNA’s we have sub-cloned into expression vectors to allow the proteins to be over-expressed in E. coli cultures, and then purified by affinity chromatography methods.

All NOS enzymes catalyze a reaction that is complex and bio-
chemically novel: the conversion of L-arginine to NO and citrulline (Scheme 1). We are currently working out the reaction mechanism of NOS through a variety of strategies. These include utilizing chemically modified substrates, intermediates, and cofactors, techniques such as Resonance Raman and electron paramagnetic spectroscopy, protein crystallography, rapid mixing stopped flow technology, and single turnover studies to probe the enzyme’s active site chemistry. We also are analyzing subdomains within the enzyme (for example, those involved in calmodulin, flavin, or heme binding) by site-directed mutagenesis and crystallography. Ongoing protein mutagenesis projects are based on our crystallographic information and include mapping the surface residues that may guide electron transfer between the subunits of NOS, identifying catalytically important residues in the active site or residues that enable enzyme structural changes in response to bound essential cofactors such as tetrahydrobiopterin (H4B) or calmodulin, and probing NOS protein elements that we think control dynamic motions of the enzyme subdomains to control or gate electron transfer within the enzyme. Some of these research topics are unique to NOS enzymes, but most are of wider relevance and of current interest to scientists who study heme proteins, flavoproteins, calmodulin, and general processes like enzyme catalysis or electron transfer.

Some Current NOS structure/function projects

Mechanisms of O2 activation by the NOS heme-
We are currently focusing on the W188H iNOS mutant, which we found stabilizes what we think is an elusive “compound 1-like” heme-oxy species, that is thought to be the ultimate oxidant in the reactions of NOS and in cytochrome P450 enzymes in general (Figure 1). We are working to better characterize the intermediate, profile its reactivity, create double mutants that should stabilize the intermediate even better, and use this mutant as a tool to understand electron donation by the H4B cofactor in NOS during NO synthesis, which can be transient and difficult to follow.

Developing and testing a global model for NOS catalysis-
NOS enzymes are unusual because they are heme proteins that generate a heme poison (NO) as their product. We have found that NOS enzymes allow the newly-formed NO to bind to the enzyme heme group before the NO is released from the enzyme. This sets up an unusual catalytic cycle that includes some heme-NO bound states of the NOS enzyme (Fig. 2). The model we developed has been very useful in understanding the activity of NOS enzymes in relation to biological O2 concentrations, for generating “super-NOS” variants by protein engineering, and for understanding the sometimes puzzling behaviors of NOS mutants. We have been measuring the kinetics of each of the separate reactions that take place during the catalytic cycle, and now we are focusing on what NOS protein structural elements control the rates of three key parameters in the NOS catalytic cycle (Fig. 2): These are

Fig. 1. Model for the oxygen activation cycle in NOS enzymes.

Fig. 2. A. Global model for the NOS catalytic cycle. B. Distribution of 3 NOS enzymes during NO synthesis.
the rates of heme reduction (kr), NO dissociation from the heme (kd), and reaction of the NOS ferrous heme-NO complex with O2 (kox). We are testing the effects of point mutagenesis and looking for NOS protein binding partners or post-translational modifications (like protein phosphorylation) that may alter the values of kr, kox, and kd in a NOS.

Mechanisms controlling electron transfer in NOS-
We are examining what controls the motions and reactivity of the FMN subdomain in NOS enzymes, because this subdomain acts as both an electron acceptor and donor at distinct points during the NO synthesis reaction (Fig. 3). We have developed ways to measure conformational states of the FMN subdomain, and are working to develop ways to do this by single molecule spectroscopic techniques that rely on fluorescent molecules being covalently and precisely linked within the NOS proteins (Fig. 4).

Characterization of the B. anthracis (anthrax) NOS-
Our collaborators in New York (Nudler lab) recently discovered that the NOS protein expressed in the anthrax bacillus was essential for its virulence. We have since expressed and purified the anthrax NOS protein and are investigating its reaction mechanism and kinetics, and will attempt to develop specific inhibitors via a high-throughput robotic drug screen available through NIH.

Some Current NOS cell biology projects

Protein-protein interactions that regulate NOS enzymes-
It’s clear that NOS activity is naturally manipulated in cells by protein-protein interactions. Our project seeks to understand at a molecular level how NOS interactions with other cellular proteins such as caveolin or heat shock protein 90 modulate the NOS activity (Fig. 5). In one approach, we have expressed and purified the active fragments or subdomains of the various partner proteins and NOS enzymes, and are studying their binding interactions and protein conformational changes by a variety of methods available at

Fig. 4. Location of the two Fluor labeling positions (yellow) on the FMN module relative to the FMN cofactor (green) and the electronegative surface patch (red).
Stuehr`s Lab.

CCF. Including protein crystallography, surface plasmon resonance (Biacore), isothermal titration calorimetry, and hydrogen-deuterium mass spectrometry. We also plan to include an $^{15}$N NMR approach when possible.

**Protein nitration**
Many questions remain about the role of cellular protein nitration: What protein components participate, how nitration can be target-specific and be selectively blocked, whether nitration causes a change in protein function, what enzymes may remove or reduce the nitro group after it is covalently attached to proteins, and the role of this process in health and disease. Within this context, we have developed proteomic methods to identify nitrated proteins within cells, and are recently focused on the subsets of cellular or mitochondrial proteins that become nitrated in response to high glucose (diabetes models), and platelet proteins that become nitrated in response to platelet activation.

**Heme insertion into soluble proteins**
The cellular biology of heme insertion for soluble protein targets is surprisingly unclear. We are using NOS and Hb as targets to study mechanisms of heme insertion into soluble proteins in cells. We have developed the means to build up heme-free NOS & Hb in cells, and then to provide heme and monitor its insertion by several means within a few hours time window. We are testing how NOS intracellular location impacts heme insertion, how NO can reversibly block heme insertion, and have discovered a few cellular proteins to be involved in the heme insertion process. We ultimately want to understand the mechanisms and regulation of the process for a variety of cellular heme proteins, and whether it is important in disease.

**Presentations-Speakers**

**Stuehr, Dennis**

**Erzurum, Serpil**
Invited speaker at the World Congress in Northern Cyprus held November 18-24, 2008. Lectured on "Airway inflammation and neovascularization" and "Free radical mechanisms in asthma Pathobiology."

**Dweik, Raed**
Symposium Director for the 5th Annual Pulmonary Hypertension Symposium: A Practical Approach to Diagnosis and Management on November 8, 2008.

**Nagy, Laura.**
Invited speaker at the Association for the Study of Liver Disease in San Francisco held November 2008. She lectured on "Pathophysiology" as part of the "Alcoholic Liver Disease" session. Clinical and basic investigators attended from around the world.
Awards

Comhair, Suzy

Received the 2008 Cleveland Clinic Innovator Award for human lung cell culture.

Kirwan, John

CTSA Pilot Grant Award for “Insulin Resistance: Detection of Hepatocellular Lipid Sub-Species by MR Spectroscopy.” This grant will develop and validate a new methodology to measure saturated and unsaturated lipid in the liver of patients with hepatic steatosis, using non-invasive NMR spectroscopy.

Awards to Trainees:

Outstanding Cooperative Education Student in Science Award, University of Limerick, Ireland, 2008. Susan O’Carroll, undergraduate student in Sports Science. For her work as a student intern in the Department of Pathobiology at the Cleveland Clinic.


Kalhan, Satish

Awarded one of this year’s two awards for “Lifetime Achievement in Diabetes Research” by The Dietrich Diabetes Research Institute of the Diabetes Association of Greater Cleveland. The Award honors select scientists, researchers or physicians who have significantly contributed to improving the quality of life for those with diabetes. Dr. Kalhan’s research focus is on understanding and managing changes in metabolism that occur in mothers with gestational diabetes and on the consequences of this condition to the fetus and neonate. He has helped develop nutritional intervention strategies for the care of premature infants. Investigating hypoglycemia in infants of diabetic mothers, he and a Case colleague invented a novel, safe means of checking blood glucose levels in the babies using isotopic tracers. The 2008 Award recipients were honored at the 3rd Annual DDRI Chairman’s Forum on Diabetes Research on October 16th.

Upcoming Department Seminars

March 3, 2009-TBA
Bela Anand-Apte, M.D., Ph.D
Cell Biology, Ophthalmic Research
Cole Eye Institute

March 10, 2009
Karen Kelly, Ph.D., Postdoctoral Fellow in Pathobiology and Nutrition

March 17, 2009-TBA

March 24, 2009
Yvonne T. Maddox, Ph.D.
NICHD Deputy Director
National Institute of Health

April 7, 2009
Sarkis Mazmanian
Assistant Professor
Biology
California Institute of Technology
Potential stem cell treatment in pulmonary hypertension (PH) is gaining increasing attention. Studies suggest that while several types of vascular stem-like cells may be involved in PH, they apparently do not all have similar roles in the disease. Definitions help us understand the types of stem cells and their potential uses in therapy. A stem cell can be likened to an ancestor cell, which then gives rise to many mother cells, called progenitors, which further expand into daughter cells.
that develop different and specialized functions. Intensive research in cardiovascular diseases has focused on the use of both vascular stem and progenitor cells for the regeneration of new blood vessels and heart muscle in injured myocardium. In humans, the peripheral blood is our major source to study endothelial stem and progenitor cells in PH. There are three types of stem or progenitor cells in the blood circulation. First is the Endothelial Colony Forming Cell (ECFC), which is considered a true endothelial stem cell. These cells are rare in the circulation (~1,000 total in an adult blood circulation), but can be expanded numerous times when needed as the building blocks of blood vessels. Thus, it is logical to propose that healthy ECFCs could be potentially helpful to rebuild the lost microvessels in the lungs of patients with PH, but currently little is known about ECFC in PH. In a study of a very small group of patients, circulating ECFC numbers were no different than found in healthy individuals. Further functional analysis of ECFC in PH is currently ongoing.

The second type is the endothelial progenitor cell, which is also found in the circulation. Unlike ECFC, these are bone marrow-derived cells, which are identified by cell-surface protein markers CD34 and CD133. These cells are present in the circulation at low levels, and in culture give rise to daughter endothelial-like cells or Colony-Forming Units of Endothelial Cells (CFU-EC). Although still relatively rare, these bone-marrow stem cells are much more commonly found in the circulation than ECFC (~2,000,000 in an adult blood circulation). The CFU-EC have the ability to contribute to repair of blood vessels, and are actually believed to be the first cells to arrive to the sites of vascular injury. The current paradigm suggests that these cells act fast by providing temporary repair and subsequently release signals that call to the ECFC, which arrive to the site and start the process of long lasting regeneration. Our recent research indicates that the circulating bone marrow CD34/CD133 progenitor cells are increased in the circulation of patients with PH as compared to healthy individuals. In fact, there are some suggestions that these cells may contribute to PH by facilitating excessive blood vessel proliferation and abnormal repair, or remodeling. Therapeutic strategies that affect bone marrow cell proliferation and release and thus inhibit these progenitor cells are currently in the pipeline. The third type of endothelial progenitor-like cell in the blood circulation is the Circulating Angiogenic Cell (CAC). These cells are derived from monocytes, bone marrow mononuclear cells that do not expand and thus are not true progenitors. However, CAC can serve similar roles in vascular health as CFU-EC. CAC are present at normal levels in the circulation of patients with PH. Because of the limited expansion capacity and their contribution to vascular health, these cells have been an attractive target for use in a combined cell – gene therapy approach for PH. Scientists have over-expressed the gene that produces nitric oxide, a molecule that affects blood vessel growth and vasodilatation, in CAC that are then infused into patients with PH. Very early stages of clinical trials are ongoing, but studies in animal models of PH suggest this may be a promising approach. Further information about this clinical trial can be found on the clinicaltrials.gov website (http://clinicaltrials.gov/ct2/show/NCT00469027?term=pulmonary+hypertension+stem+cells&rank=1). Non-endothelial stem cells are recently also being considered as transporter cells for gene therapy in PH.

Overall, the many new developments in stem cell research and PH are highly promising, but yet in early days. The contributions of each of the types of vascular stem and progenitor cells to PH are still not known, and this is important to understand for optimizing approaches. On the other hand, while there is still substantial work ahead of us before stem cells enter into standard practice, safety and efficacy clinical trials are ongoing, and should provide a wealth of information relevant to patient care soon.
New Employees

Jean Paul Achkar
Joint Appointee. His primary is in Gastroenterology & Hepatology

Nicole Fennell
Department Coordinator, in Pathobiology Administrative Offices

Dr. Kenneth McCurry
Joint Appointee. His primary is in Heart and Vascular.

Dr. Mitch Olman
new department Staff. His lab and office are located in the northeast corner of NC.

Ramasamy Somasundaram, (Rams), Postdoctoral fellow working in Dr. Stuehr's Lab

Yu Yang (Yang), Research Technologist in Dr. Dasa rathy's Lab.

Sara Haserodt, Technician in Dr. Dweik's Lab

Alquam Masir, Technician in Dr. Dweik’s Lab

New Publications-Editor

Aronica, Mark

Aytekin, Metin

Comhair, Suzy

Dweik, Raed
Featured in the article Scientists Seek to Sniff Out Diseases Electronic "Noses" May Someday Be Diagnostic Tools. M. J. Friedrich, JAMA. 2009; 301(6):585-586. The article notes Dr. Dweik’s research regarding exhaled breath analysis. “The colorimetric sensor array, which is the size of a desktop telephone, consists of 36 different chemically sensitive spots (metalloporphyrins) embedded on a disposable cartridge. Each sensor, or spot, contains different compounds that react to breath in different ways, and the color of each spot changes based on the chemicals in the breath that it interacts with, explained Dweik.”

Erzurum, Serpil

Kirwan, John

Stuehr, Dennis

Verbic, Mary Ann
P. P. Aung*, G. Murugesan, L. Zhang, J. Barmand, M. A. Verbic(1), Y. Hu, Q. Wang, K. Kotlike-Marchant. P2RY1 Gene Polymorphisms are Associated with Aspirin Resistance in Patients with A Family History of Myocardial Infarction. 1)Clinical Pathology, 2)Quantitative Health Sciences, 3)Cardiovascular Medicine, 4)Pathology and Laboratory Medicine, Cleveland Clinic, Cleveland, United States.

To learn more, visit our department on the web: http://www.lerner.ccf.org/pathobio
designed by Metin Aytekin