The 2006 Thomas Willis Lecture

The Adventures of a Translational Researcher in Stroke and Migraine

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It is especially meaningful to receive an award honoring Thomas Willis. Besides making seminal contributions to the physiology of the great circle, Willis was the first to assign separate functions to distinct brain regions and the first to number the cranial nerves in the way we identify them today. Willis was also among the earliest translational researchers, although it took >400 years for the term to emerge in our lexicon. Broad in its meaning, translational research can be highly focused and approached from bench to bedside and from bedside to bench. Bidirectional approaches are among the most efficient ways that physician-scientists can participate in the quest to discover new diagnostics and novel treatments.

So the Willis lecture will begin by providing a brief historical description of my laboratories’ contributions to research on the circle of Willis. Part 2 will focus on research advances to enhance brain perfusion by targeting the endothelium made by my colleagues and me during the past 15 years. Both areas of investigation suggest the importance of taking approaches that promote bidirectional research.

Research Contributions to the Circle of Willis

In the late 1970s, we set out to identify the sensory innervation to the circle of Willis in the hope of finding a common pain pathway relevant to migraine and stroke. Penfield, McNaughton, Wolff, and others all wrote about Willis’ circle, but not all agreed about the possibility or the importance of its sensory innervation. However, on the basis of experience in the clinic, I was convinced that the strictly unilateral headaches often reported by patients after proximal middle cerebral artery occlusions, expanding aneurysms, or migraine were sufficient to initiate studies to identify anatomic connections between the trigeminal nerve and the circle of Willis. This research program started in 1979 with a simple hypothesis in which we posited the potential existence and importance of this pathway, “neurotransmitters and the fifth cranial nerve: is there a relation to the headache phase of migraine?”1

The research began shortly after that article appeared and was accomplished by Marc Mayberg, MD. To map the innervation, he topically applied a tracer protein, horseradish peroxidase, around the middle cerebral artery. The tracer protein is taken up by axons and nerve endings and transported retrogradely to cell bodies. We hypothesized that the tracer protein would appear within neurons of the ophthalmic division. My collaborator, Robert Langer, PhD, provided a method to restrict this tracer protein to the wall of the blood vessel and thereby to avoid its diffusion throughout the subarachnoid space. We applied a novel, slow-release, polymer-based delivery system that was applied and secured to the middle cerebral artery.2 The experiment then became straightforward. Within 10 days, trigeminal neurons projecting their small, unmyelinated axons to the ipsilateral middle cerebral artery were labeled with axonal tracer (Figure 1). A novel ipsilateral pain pathway was thereby identified.3 Equally important, the application of this polymeric delivery system was among the first to demonstrate that drugs or proteins could be released in a controlled way into tubular structures such as blood vessels. This approach antedated by several years the discovery of intravascular devices with capabilities of releasing and locally restricting drugs from, eg, coated stents or other drug-releasing implants that are now placed routinely in vascular structures throughout the body.

So, the discovery of a new anatomic pathway presented the opportunity to identify its neurotransmitters, vasoactive neuropeptides (Figure 2). We and others detected substance P and calcitonin gene–related peptide, powerful vasodilators, stored in small, unmyelinated axons (C-fibers, A-delta fibers) surrounding vessels and in nerve endings within the brain stem.4–6 With noxious stimulation, peptides are released at both meningeal and brainstem sites. Our second task was to address the possible receptor sites for ergot alkaloids and triptans, drugs used in the abortive treatment of migraine. In addition to the well-known sites on vascular smooth muscle, we discovered the expression of novel serotonin, or 5-HT, prejunctional receptors on trigeminovascular axons.7,8 Occupancy of these serotonin receptors by abortive drugs inhibits
neuropeptide release and by so doing, blocks meningeal and downstream central peptidergic receptor–mediated actions that are important to headache pathogenesis.

The resulting formulation provided a “road map” that has led to the identification of several novel therapeutic targets. For example, it led to the discovery of a selective agonist activating 5-HT₁F receptors expressed by trigeminovascular axons. This agonist, like the triptans, inhibits neuropeptide release after binding to its 5-HT₁F receptor and aborts headaches, but it does so without constricting vascular smooth muscle in cerebral or coronary vessels.9 It also led to the discovery of a nonconstricting peptide receptor antagonist that blocks calcitonin gene–related peptide.10 The success of these drugs in early clinical trials established proof of principle, difficult but essential to translate into humans.

During the past 8 years, our focus moved “upstream” to address potential triggers of trigeminovascular activation and causes of migraine headaches. We provided evidence from functional imaging studies that cortical spreading depression (CSD), a slowly spreading neuroglial depolarization, was the most likely cause of migraine aura11 and such a trigger. According to this scheme, those trigeminovascular axons overlying affected brain tissue become depolarized and sensitized to cause headaches. The discharge of small, unmyelinated C-fibers may be caused by products released from neurons and glia during CSD, such as potassium, protons, or nitric oxide (NO), to then discharge trigeminovascular fibers, but these details have yet to be worked out. Nevertheless, CSD was sufficiently noxious to activate a trigeminal-autonomic reflex that depended on activation of inputs to the trigeminal nucleus caudalis and superior salivatory nucleus to cause vasodilation of blood vessels within the dura mater.12

Building on the acute models, our most recent and exciting discovery was that the most commonly used migraine prophylactic drugs suppress the susceptibility to CSD. The threshold for activation was determined by both chemical and functional imaging studies that cortical spreading depression (CSD), a slowly spreading neuroglial depolarization, was the most likely cause of migraine aura and such a trigger. According to this scheme, those trigeminovascular axons overlying affected brain tissue become depolarized and sensitized to cause headaches. The discharge of small, unmyelinated C-fibers may be caused by products released from neurons and glia during CSD, such as potassium, protons, or nitric oxide (NO), to then discharge trigeminovascular fibers, but these details have yet to be worked out. Nevertheless, CSD was sufficiently noxious to activate a trigeminal-autonomic reflex that depended on activation of inputs to the trigeminal nucleus caudalis and superior salivatory nucleus to cause vasodilation of blood vessels within the dura mater.12

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electrical stimulation paradigms. The numbers of CSDs evoked by continuous potassium application are clearly less after long-term treatment with these prophylactic drugs (Figure 3). The effects were time dependent and enantiomer specific, suggesting more than a nonspecific effect. To our knowledge, these results provide the first coherent action that may explain the common prophylactic effect in migraine of such pharmacologically diverse drugs.

Of relevance to stroke, CSDs have now been convincingly documented in the human cortex after trauma or hemorrhage, and CSDs accelerate damage in compromised tissue after experimental ischemia. So, an important challenge now is to decipher a molecular and cellular action for the prophylactic drugs and to apply that knowledge to discover new therapies in migraine and perhaps stroke.

Of course, there is much more to learn about the rare but yet-instructive connection between migraine and stroke, such as the role of sex hormones, patent foramen ovale, and silent posterior fossa lesions. For example, we now know that sex hormones modulate the susceptibility to CSD in female mice and also target the vascular endothelium. It is a reasonable guess that CSD and other slowly propagating brain events can be initiated by circulating chemicals or microcirculatory disturbances within the brain, perhaps from aggregating platelets or chemicals unfiltered from the lung.

Looking back, the focus of our studies (1) on the cerebral blood vessel, and its mechanisms of vasodilation; (2) on vessel interactions with cellular mediators released from blood vessels, from surrounding nerves, and from brain tissue; and (3) coupled with the emerging importance of CSD and its relation to astrocytes, neurons, and endothelial cells, components of the neurovascular unit, all served as a launching pad for work in translational stroke research and strategies to enhance perfusion to diminish ischemic injury.

**Research Contributions to the Endothelium and Brain Perfusion**

In the 1980s, NO was first identified in mammalian tissues by 3 future Nobel laureates. Its discovery caught my attention initially because NO is a by-product of nitroglycerin, a well-known headache inducer, but more important, because NO potently relaxes blood vessels.

After synthesis by the endothelium, NO diffuses to adjacent smooth muscle. Importantly, NO is also synthesized within neurons, and at least 2 constitutively expressed enzymes have been identified, 1 in endothelial cells (as mentioned) and 1 in neurons, each encoded by a distinct gene. When made in large quantities within neurons, NO causes cell death, in part, due to its ability to generate highly reactive oxidants. An inducible isoenzyme can also generate large quantities of NO. So there was good reason to implicate NO in ischemic pathophysiology and to consider it a potential drug target.

We leveraged this information in 5 different ways (Figure 4) that focused on endothelial NO synthase, or eNOS. Our first 3 experiments, with L-arginine and knockout mice, were designed to establish that generating NO in cerebral blood vessels was a useful target for stroke. Later experiments then tested several approaches, such as statins and Rho-kinase (ROCK) inhibitors, that were expected, based on preclinical data, to upregulate eNOS activity in experimental models and possibly in stroke patients.

In our initial step, we infused the NO precursor L-arginine and by so doing, provided, as it turns out, additional substrate for eNOS enzymatic activity. By making more NO, L-arginine potently dilated normal pial blood vessels and increased perfusion, and this also occurred in acutely ischemic tissue when given early. Figure 5 shows that L-arginine infusion increases blood flow significantly within the vulnerable peri-infarct zone after middle cerebral artery occlusion. As blood flow rose to >33% of baseline, neuronal activity recorded from the same area increased its amplitude toward normal. So, these and other experiments demonstrated the positive impact of increasing vascular NO and vasodilation on the recovery status of ischemic tissue.

On the basis of the L-arginine story, we searched for a second and different line of laboratory evidence. We turned to...
mice genetically engineered to lack the eNOS gene and tested outcomes after middle cerebral artery occlusion. In eNOS-knockouts, infarct size was larger than in controls.\textsuperscript{25} We now know that this outcome was due partly to defective vasodilation and decreased collateral blood flow. More than likely, a lack of other well-known NO actions played a role, such as decreased platelet aggregation and decreased white cell sticking within injured vessels.

In the third approach, we studied a mouse strain that was mutated to express only the eNOS isoform.\textsuperscript{20,26} When we administered to this mouse a chemical inhibitor to block expressed eNOS enzymatic activity, the lesion size grew very large. So, the results of all 3 lines of investigation were consistent with the conclusion that NO generated by the endothelium was protective during acute stroke.

We then began searching for drug strategies that might upregulate eNOS and generate NO in a more sustainable fashion than L-arginine infusion. In 1996, Matthias Endres, a postdoctoral colleague in my laboratory, began collaborating with Jim Liao’s group at the Brigham and Women’s Hospital. Liao found that statins, known as powerful cholesterol-lowering drugs, increased eNOS when added to cultured endothelial cells.\textsuperscript{27} When Endres injected statins into experimental animals, eNOS protein increased within the vascular endothelium.\textsuperscript{28} Long-term simvastatin treatment dose-dependently decreased injury after middle cerebral artery occlusion in wild-type mice with normal cholesterol levels. In fact, nearly all statins tested so far protect the ischemic brain and typically when given for periods too short to lower normal cholesterol levels.\textsuperscript{29} Because statins did not offer such protection in knockout mice lacking eNOS expression, infarct sparing in this particular model of ischemia appeared dependent on eNOS expression and clearly not on cholesterol levels.

After publication of our initial article on daily administration, other laboratories reported that short-term statin admin-

Figure 4. Five different ways to investigate the role of endothelial NO synthase and vascular NO on vasodilation and the recovery of ischemic tissue: (1) infusion of the NO precursor L-arginine dilated pial blood vessels resulting in increased perfusion. (2, 3) eNOS knockout mice develop larger infarcts attributable partly to defective vasodilation and decreased collateral blood flow. On the other hand, gene-deletion of nNOS confers neuroprotection. Inhibition of expressed eNOS in these mutants increased infarct size. (4) Statins increase eNOS activity within the vascular endothelium and decrease infarct size by a cholesterol-independent mechanism. (5) Rho-kinase inhibitors protect in experimental stroke models via eNOS-dependent mechanisms.

Figure 5. Infusion of the eNOS substrate and NO precursor L-arginine increases cerebral blood flow within the vulnerable peri-infarct zone after middle cerebral artery occlusion (MCAO). The suppressed amplitude of the electrocorticogram after MCAO, reflecting increased neuronal activity, increased towards normal after the L-arginine–induced blood flow rose.\textsuperscript{24}
istration reduces infarct size in experimental animals and also that short-term statin therapy protects in models of ischemic injury and vasospasm after subarachnoid hemorrhage.30

We now know from a large number of studies that statins impact blood vessel structure and function by several cholesterol-independent mechanisms. For example, they (1) improve endothelium function, (2) exert powerful anti-inflammatory and immune system modulatory effects, (3) reduce cytokine expression, (4) decrease oxygen radical generation, (5) exhibit antithrombotic and profibrinolytic activity, and (6) possibly even stabilize atherosclerotic plaques and their fibrous caps. Longer term, the statins have been implicated by other laboratories in angiogenesis as well as neurogenesis and synaptogenesis.31

Clinical Evidence

Could it be that the celebrated cholesterol-lowering mechanisms, as well as the so-called pleiotropic statin actions, are both therapeutically relevant? Up until fairly recently, the data linking stroke risk to elevated blood cholesterol was weak. That evidence is now getting stronger. Reducing LDL cholesterol levels turns out to be a good marker of stroke risk reduction, as evidenced by the results from the SPARCL Trial.32 So, the original reason to implicate noncholesterol mechanisms to explain a decreased stroke risk may no longer exist.33 Nevertheless, there is mounting preclinical evidence that the cholesterol-independent effects are robust, and these also appear clinically relevant. For example, there is a phase II randomized, placebo-controlled trial published in 2005 from Cambridge, England.34 This trial found that short-term statin administration markedly reduces delayed ischemic stroke and decreased vasospasm (by 80%) in patients with subarachnoid hemorrhage. Because treatment was given for 14 days only, we interpret these and 2 other preliminary studies as evidence favoring cholesterol-independent actions.8,35,36

In focal ischemia, the clinical evidence is still very preliminary. Three studies have shown that longer-term statin users appear to recover better with reduced mortality after stroke or that treatment beginning after stroke may improve patient outcome. Overall, the results, though far from “bulletproof,” favor a statin treatment effect in acute conditions within specified clinical populations, particularly in recent 2005 studies.37–39

So, how do statins exert many of their cholesterol-independent effects? One important mechanism involves the cholesterol pathway and the biochemical step normally catalyzed by the target of statins: the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase. The lipid intermediates, such as farnesyl pyrophosphate and geranyl-geranyl pyrophosphate, are key regulators of proteins like Rho and its major effector, ROCK40 (Figure 6). Rho and ROCK normally regulate endothelial cell shape change, motility, proliferation, and apoptosis, and Rho is an extremely important cell-signaling molecule. Increasing the synthesis of these intermediates increases the active form of Rho, and ROCK activity increases, thereby leading to, eg, (1) downregulation of eNOS, as found by my colleague Jim Liao; (2) unfavorable effects on the endothelial actin cytoskeleton; and (3) ROCK-mediated increases in, eg, vascular smooth muscle tone.41,42,43

The statins act by decreasing the availability of upstream intermediates. This is a consequence of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition. Decreased intermediates render Rho and ROCK less active, and this upregulates eNOS, along with several other cholesterol-independent actions. Combined with a second signaling mechanism implicating phosphorylation by other kinases such as Akt, eNOS protein and activity are both increased after statin use.44

So, based on this pathway, the prediction and the results indicate that ROCK inhibitors protect in experimental stroke models. One such inhibitor, hydroxyfasudil, appears to shrink the territory of diminished blood flow within the ischemic lesion, and eNOS is an important mediator.45 In Figure 7, one can easily see the smaller core territory and treatment effect in these laser speckle images of a treated animal versus control.

ROCK inhibitors may have a bright future, as they are now used in the clinic in Japan for treating vasospasm after
subarachnoid hemorrhage and are under administrative re-
view for the treatment of ischemic stroke. Greater blockade of
ROCK enzymatic activity may offer certain advantages over
the statins. Not surprisingly, the affected pathways are also
diverse. For example, ROCK inhibitors (1) promote regression
of atherosclerosis in experimental models and (2) slow
progression as well as (3) attenuate spasm or constriction in
coronary and cerebral vessels in patients more specifically
and perhaps more powerfully.

So, the focus on cerebral blood vessels is still evolving,
with an expanded list of targets that may one day further
amplify and complement or even replace some of the statin
actions. Among the “take-away” messages from this research
are that (1) blood vessels can be targeted for tissue protec-
tion, (2) they can be modulated at multiple sites within the
endothelium and probably vascular smooth muscle for ther-
apic benefit, (3) improving blood flow to the ischemic
lesion early on is beneficial, either by reperfusion via
thrombolysis and/or by enhanced collateral blood flow, and
(4) that targeting multiple pathophysiologic events or sites
both temporally and spatially with a single, pleiotropic drug
(analogous to combination therapy) may be particularly
advantageous for complex and evolving processes like stroke.
Clearly Rho, ROCK, and the cholesterol pathway are major
vascular regulators during health and disease and targets for
stroke prevention and treatment.

**Summing Up**

In summary, my personal view is that we are not “lost in
translation.” However, the process from theory to practice or
from pathophysiology to drug discovery is painfully slow.
Nevertheless, drug discovery could be accelerated in this
millennium by assembling large teams of basic, translational,
and clinical colleagues in academia and industry who work
together to identify targets and participate in key discoveries
from the get-go, and that means organizing a complex
infrastructure to accelerate the pace of both discovery and
application expected and, in fact, demanded by society. I
believe this challenge can be met.

So let us return to the era of Thomas Willis. In the early
17th century, great changes were taking place in science and
medicine, as they are now. Physicians in the early 1600s were
becoming less dependent on the teachings of the ancient
Greeks. Vesalius had rejected much of the practices of
Hippocrates and Galen. He relied on his own observations to
write 7 books that provided a cornerstone for modern medi-
cine during the time of Thomas Willis. Today, the corner-
stone relies increasingly on the deciphering of codes in real
time, with the emerging importance of the “omics” and
multimodal imaging. As in the century of Thomas Willis, we
are now at the dawning of a new age, and its metric will
depend on how far and how fast we advance new discov-
eries to treat human disease in this rapidly evolving field of
translational medicine.

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None.

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