Translation of Ischemic Preconditioning to the Patient
Prolyl Hydroxylase Inhibition and Hypoxia Inducible Factor-1 as Novel Targets for Stroke Therapy

Rajiv R. Ratan, MD, PhD; Ambreena Siddiq, PhD; Leila Aminova, PhD; Philipp S. Lange, MD; Brett Langley, PhD; Issam Ayoub, MD; JoAnn Gensert, PhD; Juan Chavez, PhD

Abstract—Effective therapies for stroke must interdict multiple parallel and sequential pathophysiological events. A paradigm which offers insight into multivalent but thoughtfully coordinated protective programs is ischemic preconditioning. A central hypothesis of our group and others is that pharmacological agents that activate programs of gene expression normally induced by ischemic preconditioning will be effective agents for the prevention and treatment of stroke. Inhibitors of a class of enzymes, the hypoxia inducible factor-1 (HIF-1) prolyl hydroxylases stabilize the transcriptional activator HIF-1 and activate target genes involved in compensation for ischemia, including erythropoietin (Epo) and vascular endothelial growth factor (VEGF). Here, we review evidence suggesting that the HIF-1 prolyl hydroxylases are inhibited during ischemic preconditioning and that pharmacological inhibitors of these enzymes are viable targets for stroke therapy. (Stroke. 2004;35[suppl I]:2687-2689.)

Key Words: acute care ■ hypoxia ■ ischemia ■ stroke ■ transcription

While deprivation of necessary metabolic fuels is an established common final pathway to stroke, there is growing awareness of stroke as a heterogeneous disorder. Indeed, stroke can be ischemic or hemorrhagic; it can involve interruption of small or large vessels involving intracranial or extracranial arteries or veins; it can be exacerbated by hypotension, fever, or hyperglycemia; it can be influenced by age, gender, and racial background; and it can be influenced by comorbidities and concurrent medications.

The above list is not exhaustive, but the heterogeneity of stroke argues that one needs a therapeutic approach that recognizes and addresses the complexity inherent in its diverse causes and outcomes. One strategy to address this complexity is combinatorial therapy, which has been used successfully in the treatment of cancer, HIV infection, and tuberculosis. Indeed, several labs have begun to apply the principles of combinatorial therapy to stroke, with promising preclinical results.

An alternative to combinatorial therapy is to identify single agents that act on a multivalent, but thoughtfully coordinated, homeostatic response. Such a coordinated response would involve multiple gene programs (eg, survival, repair, and revascularization) that act in various cell types (neuronal, glial, and endothelial). A biological paradigm that provides insight into multivalent protective responses in the brain is ischemic preconditioning. Ischemic preconditioning is a fascinating biological phenomenon in which exposure of animals (and likely humans as well) to a short, sublethal ischemic insult provides immediate and lasting resistance to a subsequent, more severe insult. The lasting resistance appears to result in part from the de novo expression of genes involved in homeostatic responses to hypoxia and ischemia. A central working hypothesis of our group and others is that pharmacological agents that activate programs of gene expression normally induced by ischemic preconditioning will be effective agents for prevention and treatment of stroke.

Hypoxia Homeostasis, Hypoxia Inducible Factor-1, and Ischemic Preconditioning

Oxygen is essential for metabolic processes, including oxidative phosphorylation, in which it serves as an electron acceptor during ATP formation. Over the past 15 years, a host of cellular, local, and systemic homeostatic mechanisms have been shown to be activated by hypoxia, defined as the state when oxygen demand exceeds supply. An example of hypoxia homeostasis includes erythropoiesis by individuals who are anemic or who live at high altitude. Erythropoiesis is the result of upregulation of the growth factor erythropoietin (Epo) and serves not only to increase red blood cell mass, but also to protect the brain. Another example of hypoxia homeostasis is neovascularization in the hypoxic brain. This appears to be mediated via the transcriptional upregulation of vascular endothelial growth factor (VEGF). VEGF not only has neuroprotective properties, but it also can
stimulate angiogenesis.\textsuperscript{13} Finally, cells cultured in vitro at reduced oxygen tension show increased glycolysis as a result of increased expression of glycolytic enzymes and glucose transporters.\textsuperscript{14} This compensatory response permits ATP generation in the absence of oxygen.

Over a decade ago, Gregg Semenza and colleagues at Johns Hopkins University Medical School used a series of transgenic mice to identify a sequence in the 3′ untranslated region of the Epo gene that is required for its upregulation by hypoxia.\textsuperscript{15} Using a portion of this sequence to make a DNA affinity column combined with industrial quantities of lysates from cultured Chinese hamster ovary cells exposed to a hypoxia mimic, the Semenza group purified a heterodimeric complex of proteins that bind to DNA to regulate hypoxia gene expression.\textsuperscript{15} They designated this complex as hypoxia-inducible factor 1 (HIF-1). It is composed of a 120 kDa HIF-1α subunit and a 91 to 94 kDa HIF-1β subunit. Established inducers of Epo expression, including iron chelators, cobalt chloride, and hypoxia, induce HIF-1. Of note, the canonical binding site for HIF-1 (5′-GCGTG-3′) is found in the promoter regions of many of the genes one would choose to compensate for hypoxia-Epo, VEGF, glycolytic enzymes, heme oxygenase, glucose transporters, neuroglobin, etc, and although Epo is limited in its expression to the kidney, brain, and brain, HIF-1 appears to be ubiquitously expressed in all tissues, including brain.

The evidence that HIF-1 is activated in response to ischemic preconditioning in brain is extensive: (1) ischemic preconditioning in the brain requires new transcription and is not dependent on the activation of glutamate receptors;\textsuperscript{16} (2) a 3-hour exposure (the duration of exposure required for ischemic preconditioning) to 8% O\textsubscript{2} induces HIF-1 in the neonatal brain;\textsuperscript{17} (3) preconditioning with pharmacological activators of HIF-1 (deferoxamine or cobalt chloride) rather than hypoxia confers significant protection in the central nervous system (CNS);\textsuperscript{17} (4) infusion of soluble Epo receptor (intracerebroventricularly) reverses the protective effect of ischemic preconditioning.\textsuperscript{18}

**Protein Hydroxylation, HIF-1α Stabilization, and Activation of a Central Homeostatic Response to Stroke**

The putative role of HIF-1 in the neuroprotective effects of ischemic preconditioning raises the possibility that small molecule “drugs” that act to stabilize HIF-1α and enhance HIF-1–dependent transcription may be viable agents for human stroke therapy. Indeed, over the past decade the signaling paths involved in HIF-1 activation have been deciphered in great detail. Under normoxic conditions, HIF-1α is constitutively transcribed and translated. However, the stability of the protein is drastically reduced by the hydroxylation of HIF-1 at prolines 402 and 564 by HIF-1 prolyl hydroxylases. Hydroxylated HIF-1 recruits the E3-ubiquitin ligase Von Hippel Lindau (VHL) protein. VHL protein tags HIF-1 with ubiquitin groups and targets it for degradation by the proteasome.\textsuperscript{19}

As expected from this model, the prolyl hydroxylases involved in regulating HIF-1α stability are oxygen-dependent.\textsuperscript{20,21} Thus, under conditions of hypoxia, these enzymes function with low efficiency, resulting in HIF-1α that is not hydroxylated. Because nonhydroxylated HIF-1α cannot interact with VHL, it becomes stabilized. Stable HIF-1α can then bind to its heterodimeric partner HIF-1β, and together these proteins can act in the nucleus to transactivate genes involved in adaptation to hypoxic-ischemic stress.

The prolyl hydroxylases not only require oxygen, but iron and 2-oxoglutarate\textsuperscript{22,23} to hydroxylate the critical prolines on HIF-1α. Iron and 2-oxoglutarate are thus rational pharmacological targets for inhibiting the HIF-1 prolyl hydroxylases and activating HIF-1 under conditions of normoxia, or for augmenting HIF-1 activation under conditions of ischemia.\textsuperscript{23} It is not surprising that some of the best-established activators of HIF-1 are chelators of iron. Indeed, previous studies from our laboratory demonstrated that 2 distinct chelators of iron, deferoxamine mesylate and mimosine both significantly induce HIF-1 and its target genes in neurons and protect neurons from oxidative stress.\textsuperscript{24} Subsequent studies by 2 other groups demonstrated that delivery of desferoxamine mesylate to neonatal or adult animals reduces infarct volume in models of focal ischemia.\textsuperscript{17} The findings provided experimental substance to the notion that inhibitors of the prolyl hydroxylase are neuroprotective.

**Which Prolyl Hydroxylase Inhibitors Will Be Best for Stroke Therapy?**

It is not clear at this time whether iron chelators will be the best HIF-1 prolyl hydroxylase inhibitors for stroke therapy. It is well established that iron is a necessary cofactor for a host of important cellular functions, including oxidative phosphorylation and arachidonic acid signaling.\textsuperscript{25} Therefore, it may be less desirable to use an iron chelator as a therapeutic for a brain disease such as stroke. By contrast, iron chelators have numerous salubrious effects on cell function via mechanisms that may be independent of HIF-1.\textsuperscript{26} Thus, despite potential toxicity, the ability of iron chelators to affect posttranscriptional and transcriptional targets may be particularly attractive in the treatment of multiple types of stroke. A systematic examination of the relative efficacy of prolyl hydroxylase inhibitors that bind iron versus those that do not is needed.

Another strategy to identify novel, safe prolyl hydroxylase inhibitors is to screen a library of drugs already approved for use in humans (FDA-approved drugs). Such a screen might elude agents that would more potently activate HIF-1 in the CNS; additionally, agents that activate HIF-1 preferentially in glia, neurons, or endothelial cells might be identified. The screen holds the obvious clinical benefit that all of the agents have passed a series of rigorous tests for safety in humans. Our laboratory has been involved in screening an FDA-approved library for novel HIF-1 activators over the past few years. A number of agents have been identified and preliminary studies are ongoing to document when, where, and how these agents can activate HIF-1 in the CNS.

**There Is Good and Bad in All of Us: HIF-1 Is No Different**

It is important to point out that while HIF-1 mediates the expression of many established neuroprotective genes, it also induces expression of prodeath genes. Some of these genes
include the prodeath Bcl-2 family member, BNIP3. Despite the association of HIF-1 with cell death in some experimental paradigms, what is important in the evaluation of HIF-1 as a target for therapy is that the net effect of HIF-1 activation be beneficial for the organism. Moreover, as novel targets for the prolyl hydroxylases have been identified (ie, IRP-2, RNA polymerase 2), it is also important to note that prolyl hydroxylase inhibition will stabilize not only HIF-1α but a host of other proteins as well. The important message for physicians who treat stroke is that pharmacological inhibition of the HIF-prolyl hydroxylases is not equivalent to HIF-1α overexpression.

HIF-1 Prolyl Hydroxylases: Promising But As Yet Unproven Therapeutics for Stroke

The identification of a panel of prolyl hydroxylase inhibitors that bind iron, displace 2-oxoglutarate, or work on other aspects of the HIF-1 pathway will provide useful tools to determine which of the available sites of pharmacological attack in prolyl hydroxylases, if any, will be most advantageous in the treatment of stroke. Important and as yet unanswered questions include the timing of intervention with these agents relative to stroke onset. Must HIF-1 prolyl hydroxylase inhibitors be given as preventative measures or can they be given after stroke? If gene expression is required for the salubrious effects of HIF-1 prolyl hydroxylase inhibitors, then it seems unlikely that the most acute injury events of stroke will be interdicted by this approach. However, given that HIF-1 prolyl hydroxylase inhibition may stabilize numerous other proteins in addition to HIF-1, it is possible, perhaps likely, that the positive effect of this approach will be immediate. Moreover, as mentioned above, the ability of iron chelators to activate HIF-1 dependent gene expression and to sequester iron that could participate posttranslationally in devastating oxidant production via reactions such as Fenton chemistry suggests that these “dirty” agents can affect multiple pathways to injury. Despite the details that have yet to be elucidated, the notion of being able to pharmacologically activate a multifaceted homeostatic response that works at a cellular, local, and systemic level to alleviate the discrepancy between substrate (oxygen/glucose) supply and demand holds tremendous intuitive appeal. It is our fervent hope that such a strategy, which takes advantage of endogenous homeostatic pathways, will be more efficacious while less toxic.

Acknowledgments

This work was supported by the National Institutes of Health and the Burke Foundation.

References

Translation of Ischemic Preconditioning to the Patient: Prolyl Hydroxylase Inhibition and Hypoxia Inducible Factor-1 as Novel Targets for Stroke Therapy
Rajiv R. Ratan, Ambreena Siddiq, Leila Aminova, Philipp S. Lange, Brett Langley, Issam Ayoub, JoAnn Gensert and Juan Chavez

Stroke. 2004;35:2687-2689; originally published online October 7, 2004; doi: 10.1161/01.STR.0000143216.85349.9e

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/35/11_suppl_1/2687

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/