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PERSPECTIVE

Potential Therapeutic Gene for the Treatment of Ischemic Disease: Ad2/Hypoxia-Inducible Factor-1α (HIF-1)/VP16 Enhances B-Type Natriuretic Peptide Gene Expression via a HIF-1-Responsive Element

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ABSTRACT

In this issue of *Molecular Pharmacology*, Luo et al. (p. 1953) present a study employing a HIF-1α/VP16 chimera to investigate the mechanism by which this constitutively active transcription factor activates expression of brain natriuretic peptide (BNP). The results define a functional hypoxia responsive element (HRE) in the promoter of the human *BNP* gene and demonstrate that this HRE is necessary for HIF-1α/VP16-induced gene expression in human cardiomyocytes grown under normoxic conditions. Luo et al. also show that a consensus E-box DNA binding sequence is necessary for appropriate *BNP* regulation. Because HIF-1 is known to elicit protective and beneficial gene expression programs in many scenarios and because BNP is known to be cardioprotective, this study provides support for the therapeutic use of the chimeric HIF-1α/VP16 protein in coronary heart disease. However, because HIF-1α is a key regulatory molecule that acts upon a large number of downstream gene networks, there remains a need for further investigation. Particularly useful would be comprehensive gene expression profiling coupled with functional analysis of HIF-1α/VP16-regulated genes. The results of such studies will elucidate the mechanism of beneficial effects and address concerns regarding potential adverse effects of activating specific HIF-1α/VP16-dependent gene programs.

Because organisms evolved first under anaerobic conditions, many conserved biological functions, including glycolysis, DNA synthesis and aspects of gene expression regulation retain the requirement for a reducing microenvironment (Segerer et al., 1985). Such "anaerobic processes" became integrated with those that subsequently evolved under aerobic conditions. In aerobic organisms, under conditions of hypoxia, defined as deficiency of oxygen in tissues, continued activation of oxidative metabolism produces increased oxidative stress and cellular damage. Therefore, mechanisms have evolved to suppress oxidative phosphorylation in favor of glycolysis under these conditions. These mechanisms include several signaling and transcription factor systems that coordinately activate glycolytic enzyme-encoding and stress response genes (for review, see Webster, 2003). These mechanisms allow cells to adapt to hypoxia, a common environmental stimulus.

Ischemia is defined as a deficiency in blood flow as a result of obstruction or reduction of arterial supply. In the heart, coronary spasm or occlusion (e.g., atherosclerosis or thromboembolism) causes ischemia and/or ischemia/reperfusion (I/R) injury. The first phase of I/R, ischemia, causes hypoxia to tissues (myocardium) downstream from the site of occlusion. During hypoxia, myocardial cells switch from oxidative to glycolytic metabolism, resulting in increased glucose utilization, lactose production and therefore low intracellular

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ABBREVIATIONS: I/R, ischemia/reperfusion; PAD, peripheral arterial disease; MI, myocardial infarction; HIF, hypoxia-inducible factor; bHLH, basic helix-loop-helix; PAS, PER/ARNT/SIM (periodicity/aryl hydrocarbon receptor nuclear translocator/simple-minded); TA, transcriptional activation; ODD, oxygen-dependent degradation domain; HBS, hypoxia binding site; HRE, HIF-1 responsive element; VEGF, vascular endothelial growth factor; BNP, brain natriuretic peptide; KO, knockout; PDGF, platelet-derived growth factor.

pH (Neely and Grotyohann, 1984; Dennis et al., 1991; Webster et al., 2000). Thus, myocardial ischemia has aspects of both hypoxia and acidosis. This combination has been shown to result in cell death associated with activation and increased mitochondrial localization of BNIP3 (Webster et al., 1993; Kubasiak et al., 2002). Reperfusion occurs when blood flow is restored and results in exacerbation of myocardial cell injury associated with increased oxidative stress and production of reactive oxygen and reactive nitrogen species (Takano et al., 2003; Zhao, 2004). Cell death in I/R injury is a mixture of apoptosis and necrosis (Holleyman and Larson, 2001). Hypoxia, ischemia, and I/R injury are relevant to a number of clinically important disorders, including stroke, peripheral arterial disease (PAD), coronary artery disease, myocardial infarction (MI), and cardiomyopathy.

Aerobic cells are optimally functional within a narrow range of tissue oxygen partial pressure (Po_2), from 50 to 70 mm Hg (Hochachka, 1999). Hypoxia initiates changes in expression of hypoxia-regulated genes that result in physiological reactions, including erythropoiesis, angiogenesis, and increased glycolysis, as well as other metabolic changes (Ikeda, 2005). These responses to insufficient oxygen levels are essential for cell survival (Jiang et al., 2002). A key player in the regulation of the cellular responses to various physiological and pathological effects of hypoxia is the transcription factor hypoxia-inducible factor-1α (HIF-1α) (Ikeda, 2005). HIF-1α, an oxygen-sensitive protein, is stabilized (i.e., activated) under hypoxic conditions but is degraded (i.e., inactivated) under normoxic conditions. Thus, HIF-1α-dependent genes are repressed under normoxic conditions (Ikeda, 2005) and activated in tissues with Po_2 levels less than 40 mm Hg (Hochachka, 1999). The central role of HIF-1α in the cellular response to hypoxia makes this factor an attractive therapeutic target for hypoxia-related pathologies (Giaccia et al., 2003). In this issue of *Molecular Pharmacology*, Luo et al. (2006) present work bearing upon the mechanism of action of a constitutive HIF-1α/VP16 protein with potential therapeutic utility.

HIF Transcription Factors

There are four known HIF family transcription factors (HIF-1α, HIF-1β, HIF-2α, and HIF-3α), all of which share the same structural organization. HIF transcription factors are DNA-binding proteins of the basic helix-loop-helix (bHLH) and Per/ARNT/SIM (PAS) families (Jiang et al., 1996; Giaccia et al., 2003). The genes encoding all four proteins are constitutively expressed, and HIF proteins bind to DNA as heterodimers, most commonly HIF-1α and the aryl hydrocarbon nuclear transporter, also known as HIF-1β. The protein domain structure for the 826-amino acid HIF-1α protein is shown in Fig. 1, and includes the bHLH domain required for protein dimerization and DNA binding. The PAS domain is required for heterodimerization and protein interaction, probably with multiple proteins. The transcriptional activation domains (TA) are near the carboxyl terminus of HIF-1α and are required for transcriptional activation of HIF-1α-dependent genes. A critical domain for HIF-1α regulation is the oxygen-dependent degradation domain (ODD), also called the hypoxia stability domain (HSD). Regardless of the active heterodimer, the stabilization of HIF-1α during hypoxia mediates the activity of HIF-dependent gene expression. Under normoxic conditions, the HIF-1α ODD domain interacts with the pVHL protein, which recruits prolyl hydroxylases that specifically hydroxylate Pro402 and Pro564 of HIF-1α (Giaccia et al., 2003; Zagorska and Dulak, 2004; Ikeda, 2005). These hydroxylations are oxygen-dependent and enhance recruitment of ubiquitin ligases, resulting in ubiquitinization of specific amino- and carboxyl-terminal regions of the HIF-1α protein. The ubiquitinated HIF-1α protein is then targeted for destruction by the 26S proteosome (Fig. 2). Under normoxic conditions, the half-life of HIF-1α is less than 10 min, and the levels of the HIF-1α protein are undetectable (Salceda and Caro, 1997; Zagorska and Dulak, 2004), preventing activation of HIF-1-dependent gene expression. An additional level of regulation of HIF-1α activation occurs by hydroxylation of Asn803, which prevents interaction of the carboxy-terminal TA domain with transcriptional cofactors such as p300 (Ikeda, 2005). All of these hydroxylations are reduced or prevented during hypoxia, resulting in stabilization and increased potency of transactivation by HIF-1 heterodimers (Fig. 2). Other than an enzymatic requirement for oxygen and a likely role for reactive oxygen species and specific protein kinase pathways, the detailed signaling mechanisms that result in oxygen sensing remain incompletely understood and have been reviewed elsewhere (D'Angio and Finkelstein, 2000; Zagorska and Dulak, 2004).

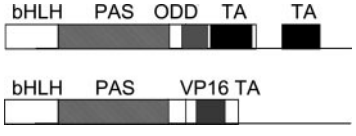

Fig. 1. Structure of HIF-1α and the HIF-1α/VP16 hybrid transcription factors. The bHLH and PAS domains are indicated. ODD is the domain for pVHL interaction (oxygen-dependent degradation domain or oxygen-ensitive domain). The HIF-1α/VP16 is a fusion of HIF-1α (amino acids 1–390) and the transactivation domain of herpes virus VP16 (amino acid 413–490) (Vincent et al., 2000).

HIF-1α is a major downstream effector of many of the signaling pathways activated by hypoxia. HIF-1α binds gene promoters via a hypoxia binding site (HBS), also known as an HIF-1 responsive element (HRE). The HBS has a consensus sequence of A/GCGTG. In some promoters, these sites are clustered; in others, HBS sites cluster with binding sites for other transcription factors. In several instances, these transcription factors bind cooperatively with HIF-1 and are necessary in combination to activate gene expression. This type of regulatory mechanism, perhaps in combination with cell-type specific expression of HIF isoforms (e.g., HIF-2α and HIF-3α) is believed to underlie cell type-specific binding and activation of HIF-1-dependent genes during hypoxia.

HIF-1α-Dependent Gene Expression. It has been shown that modulating HIF-1α activity increases cell survival after hypoxia (Chi and Karliner, 2004; Date et al., 2005). Furthermore, enhanced angiogenesis at sites of vascular disruption or dysfunction contributes chronically to the protective effect (Giaccia et al., 2003). Vincent et al. (2000) developed a construct that expresses a chimeric protein (HIF-1α/VP16) consisting of the DNA-binding and dimerization domains of HIF-1α (first 390 amino acids) and the transactivation domain from herpes simplex virus VP16 protein (Fig. 1). This chimeric protein dimerizes, binds DNA, and acts as a constitutively active HIF-1α protein. Because of the strong VP16 transcriptional activation domain, HIF-1α/VP16 is a powerful inducer of HIF-1α-dependent gene expression. Vin-

cent et al. (2000) examined the activity of the construct by measuring the expression of known HIF-1-responsive genes, including vascular endothelial growth factor (VEGF), in vitro and in vivo. The results confirmed that the HIF-1α/VP16 protein is a strong, constitutive transcriptional activator under normoxic conditions. Administration of HIF-1α/VP16 expressing constructs has been shown to increase blood flow and reduce cell death in animal models of both cardiac and peripheral ischemic disease (Vincent et al., 2000; Shyu et al., 2002). Understanding the role that HIF-1α-inducible gene expression plays in adaptive response is important for elucidating the physiological and pathophysiological mechanisms that underlie specific disease states.

Because HIF-1α is a transcription factor, its physiological and pathophysiological effects must be understood in terms of the regulation of downstream genes. Jiang et al. (2002) characterized the response to transfection with Ad2/HIF-1α/VP16 via gene expression profiling using human cardiac cell and serial analysis of gene expression. Among the genes found to be up-regulated in this study by HIF-1α/VP16 was the brain natriuretic peptide (BNP) gene. However, hypoxia alone had no significant effect upon expression of the *BNP* gene (Jiang et al., 2002; Luo et al., 2006), suggesting a difference in the transactivational capabilities of HIF-1α/VP16 versus the endogenous HIF-1α.

A hallmark of cardiac hypertrophy, heart failure, and acute myocardial ischemia is an increase in *BNP* expression levels and secretion (Kawakami et al., 2004; Ruck et al., 2004; LaPointe, 2005). The physiological roles of BNP have been studied using both *BNP* knockout (*BNP* KO) and *BNP* transgenic mice. *BNP* KO mice compared with control mice were found to have more extensive ventricular fibrosis at baseline (Tamura et al., 2000; LaPointe, 2005). *BNP* transgenic mice with elevated plasma BNP compared with control mice had a significant increase in neutrophil infiltration and matrix metalloproteinase 9 activity in the ischemic and border zones 3 days after MI (Kawakami et al., 2004). The results suggest that BNP plays a key role in the process of extracellular matrix remodeling and wound-healing after acute MI. It is clear that understanding how BNP is regulated and acts in coronary disease will contribute to the development of therapeutic strategies to prevent or reverse adverse consequences of chronic heart disease (LaPointe, 2005). In the study by Jiang et al. (2002), the mechanism that underlies the effect of HIF-1α/VP16 upon *BNP* expression was not determined.

In this issue, Luo et al. (2006) present results bearing upon the mechanism of HIF-1α/VP16-mediated up-regulation of BNP expression. The authors showed that *BNP* expression increased 24 h after transfection with an adenovirus expressing HIF-1α/VP16 in primary human fetal cardiomyocytes. Hypoxia alone or overexpression of native HIF-1α had no significant effect upon BNP levels at this timepoint. Luo et al. (2006) further demonstrated that *BNP* is up-regulated in human cardiomyocytes transfected with a virus that expresses native HIF-1α but only after exposure to hypoxia for 72 h. These results call into question whether the mechanism of *BNP* up-regulation by HIF-1α/VP16 is direct or indirect. The authors address this by transfecting cells with a HIF-1αΔ/VP16 construct that has a deletion of the DNA binding and dimerization domains of HIF-1α/VP16 (i.e., removes amino acids 24–170) and examining the effects upon *BNP* gene expression. The HIF-1αΔ/VP16 protein was unable to bind DNA and did not up-regulate *BNP* expression, demonstrating that the effect of HIF-1α/VP16 is dependent upon DNA binding via the HIF-1α component. Next, Luo et al. (2006) employed deletion analysis of the BNP promoter fused to a luciferase reporter to determine the location of the *cis*-acting sequence conferring activation by HIF-1α/VP16 in primary rat neonatal cardiomyocytes. The results of these experiments delineated a putative HRE at −466 of the BNP promoter that mediates the HIF-1α-specific effects of HIF-1α/VP16 upon BNP gene expression. Confirmatory studies showed that a 50-base-pair region (mHRE) containing this HRE confers HIF-1α/VP16 regulation of gene expression. An HIF-1α/NF-κB chimeric protein was also found to activate

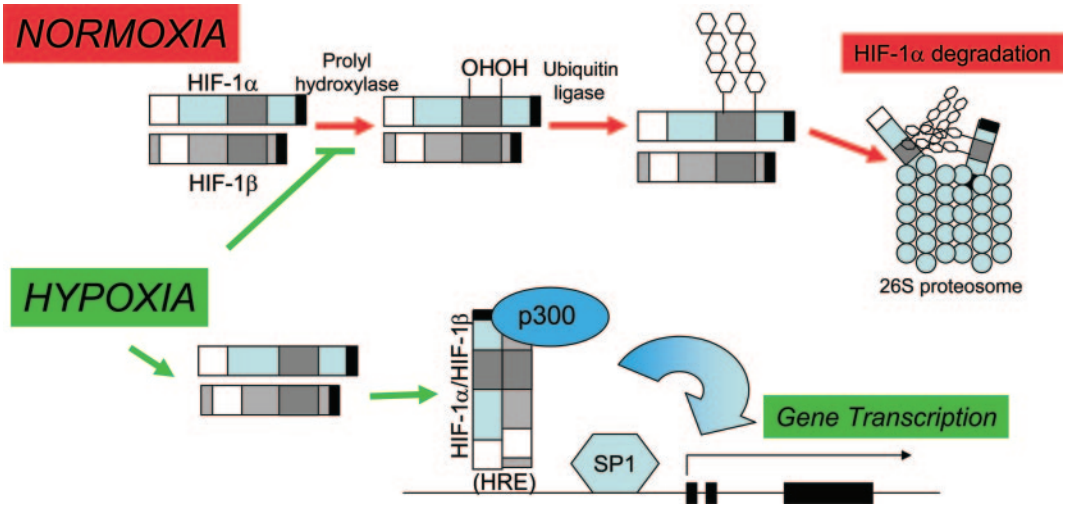

Fig. 2. Activation of HIF-1-dependent gene expression. Under conditions of normoxia, Pro402 and Pro564 of HIF-1α are hydroxylated, ubiquitinated, and the protein degraded by the 26S proteosome. Asn803 may also be ubiquitinated with effect upon transcription coactivator interaction (not shown). However, under conditions of hypoxia, HIF-1α is stabilized by repression of prolyl hydroxylases. Under these conditions, HIF-1α dimerizes with HIF-1β and can bind the HRE of HIF-1-dependent gene promoters, thereby contributing to transactivation of these promoters by interacting with the basal transcriptional machinery (including SP1). The white box on the HIF-1 molecules includes the bHLH and the PAS domains. Gray boxes, ODD; black boxes, transcriptional activation domains.

gene expression via the mHRE, showing that a different activation domain (from NF-κB in this construct) can also up-regulate HIF-1-α-dependent gene expression. Mutational analysis of the mHRE demonstrated that gene expression is dependent upon an intact HRE and upon an adjacent E-box transcription factor binding site. Finally, results of electrophoretic mobility shift assays confirm binding of HIF-1α/VP16 and HIF-1α to the HRE and mutations within the HRE prevent binding. The results shed light upon the mechanism of action of HIF-1α/VP16-induced BNP expression and add to the rationale for therapeutic use of the chimeric HIF-1α/VP16 transcription factor.

The authors address the differences between gene expression via the overexpressed HIF-1α/VP16 and the endogenous HIF-1α protein at 24 h of hypoxia. Their interpretation is that the endogenous HIF-1α does bind the mHRE in the BNP promoter, but that the sensitivity of the promoter to HIF-1α is low. This is mitigated by overexpression of the chimeric HIF-1α/VP16 protein, because there are higher protein levels and the chimeric protein has a stronger transcriptional activation domain. The result (i.e., that BNP is slightly increased via endogenous HIF-1α at a later point) suggests that long-term HIF-1α activation eventually does up-regulate BNP and that HIF-1α/VP16 serves to accelerate and enhance this activation. These results also serve as a cautionary note, because it is possible that HIF-1α/VP16 expression could result in induction of deleterious genes that are not normally expressed in an hypoxic response (i.e., not significantly dysregulated by endogenous HIF-1α). As noted by the authors, this has not yet been observed in experimental settings. However, comprehensive expression profiling in vivo has not yet been performed with HIF-1α/VP16.

Clinical Relevance of HIF-1α in Coronary Disease. Approximately 53% of deaths from cardiovascular disease are related to coronary heart disease and 13.2 million people in the United States have coronary disease (Thom et al., 2006). In addition, heart failure and stroke affect 10 million Americans annually (Thom et al., 2006). The underlying cause of both coronary heart disease and stroke is atherosclerosis, which results in arterial damage and ischemic disease. Therefore, there is a critical need to understand the molecular physiology of ischemic disease.

As previously discussed, hypoxia is a major component of ischemic disease, and HIF-1α has been shown to up-regulate expression of genes involved in protective effects, including angiogenesis, stress response, nitric oxide synthesis (i.e., inducible nitric-oxide synthase), extracellular matrix remodeling and vascular perfusion. Work employing cultured neonatal rat cardiomyocytes subjected to simulated I/R demonstrated a protective effect similar to that of late ischemic preconditioning associated with Ad2/HIF1α/VP16 infection (Date et al., 2005). Shyu et al. (2002) found that HIF-1α/VP16 reduces infarct size and increases collateral vessel formation in myocardial tissue from rats after acute myocardial infarction (Shyu et al., 2002). A more recent study using an in vivo porcine model of chronic myocardial ischemia demonstrated significant improvement in myocardial perfusion and post-ischemic left ventricular dysfunction (Heinl-Green et al., 2005). The current study by Luo et al. (2006) supports the idea that HIF-1α/VP16 is capable of up-regulating *BNP* in cardiomyocytes (Luo et al., 2006). The fact that BNP is associated with cardioprotective effects (Nishikimi et al., 2006) supports the idea that the HIF-1α/VP16 chimeric protein may be therapeutic. However, the mechanism by which HIF-1α/VP16 results in cardioprotection against ischemia and I/R injury remains incompletely understood. The current work did not demonstrate that increased BNP levels mediate protective effects. Work needs to be done to more thoroughly understand the mechanism of therapeutic HIF-1α/VP16 expression. Additional studies need to address the possibility that adverse effects may result from dysregulation of genes that are either 1) HIF-1-regulated or 2) not HIF-1-responsive but induced by the chimeric HIF-1α/VP16 protein. The potential complications of up-regulating a key regulatory factor at this stage of understanding probably precludes clinical studies in the heart at this time. Further gene profiling studies using in vivo models coupled with functional studies to link gene expression changes to beneficial and potentially adverse effects are needed.

Clinical Relevance of HIF-1α in Peripheral Ischemic Disease. Fully 7.5% of the US population aged 60 to 64 years in United States is affected by PAD, which is primarily related to atherosclerosis (Bendermacher et al., 2005; Patel et al., 2005). The most common treatment for critical limb ischemia is surgical bypass and percutaneous revascularization (Patel et al., 2005). However, many candidates are excluded from treatment because of anatomical distribution or extent of vascular disease, and a common outcome is limb amputation (Patel et al., 2005). Thus, there is a critical need for novel therapeutic strategies to treat PAD in these patients. Heretofore, strategies that have been employed include treatment with effector proteins to stimulate angiogenesis or neovascularization, including VEGF, fibroblast growth factor, and platelet-derived growth factor (PDGF). Results from these studies have been inconsistent, and the efficacy of these treatments remains in doubt (Freedman and Isner, 2001; Khan et al., 2003; Serruys et al., 2003; Gounis et al., 2005; Kastrup et al., 2005; Makino et al., 2005; Patel et al., 2005; Shah and Losordo, 2005).

Vincent et al. (2000), who first constructed the HIF-1α/VP16 chimeric protein used by Luo et al. (2006), showed that HIF-1α/VP16 induced VEGF and erythropoietin expression under normoxic conditions. It is noteworthy that Vincent et al. (2000) found HIF-1α/VP16 to be more potent than hypoxia in the induction of VEGF. These authors showed that the HIF-1α/VP16 gene significantly improved blood pressure and flow 40 days after ischemia using a rabbit hind-limb ischemia model. Analyses revealed higher capillary densities in muscle that had been transfected with the HIF-1α/VP16 gene.

A recent study (Patel et al., 2005) that used an adenovirus encoding a constitutively active form of HIF-1α (CA5, a deletion of amino acids 392–520 and two missense mutations Pro567Thr and Pro658Gln) showed increased expression of genes encoding angiogenic factors, including PDGF-B, placenta growth factor, stromal cell derived factor, monocyte chemoattractant protein-1, and VEGF. In this study, the CA5 adenovirus was delivered via intramuscular injection into a rabbit model of atherosclerotic peripheral arterial obstruction (Patel et al., 2005). The CA5-transfected rabbits showed improvement, 14 days after administration, in blood pressure, peripheral perfusion, and distal deep arterial diameter relative to rabbits transfected with a lacZ construct (Patel et al., 2005). HIF-1α regulates pro-angiogenic factors (Vincent et al., 2002; Pugh and Ratcliffe, 2003; Josko and Mazurek,

2004; Paul et al., 2004; Rebar, 2004) and protective stress response pathways, and it activates BNP (Luo et al., 2006), which can increase peripheral vascular flow (Cargill and Lipworth, 1995; van der Zander et al., 1999). Therefore, it is conceivable that HIF-1α/VP16 overexpression may provide synergistic beneficial therapeutic effects in PAD (Pajusola et al., 2005). Clinical trails using HIF-1α/VP16 for PAD are being conducted. As of this date, phase I trials have been completed and phase II trials, begun in 2005, will be completed in 2009 (http://www.clinicaltrials.gov/ct/show/NCT00117650?order = 2). Questions remain regarding the mechanism underlying beneficial effects and the possibility of adverse effects, including tumor growth (as a result of angiogenesis) and progression of diabetic retinopathy or rheumatoid arthritis (reviewed by Ikeda, 2005). However, localized delivery and transient expression of HIF-1α/VP16 may prove an efficacious alternative to bypass and amputation in patients with severe PAD.

Summary

The study by Luo et al. (2006) in this issue of *Molecular Pharmacology* use a potential therapeutic gene, currently being evaluated in clinical trails for PAD, to investigate the mechanism whereby HIF-1α/VP16 protects the myocardium against hypoxia and ischemic damage. These studies derive from the results of Jiang et al. (2002) using serial analysis of gene expression, which demonstrated that HIF-1α/VP16 expression up-regulates *BNP* in cardiomyocytes. The authors of the current article (Luo et al., 2006) define a functional HRE in the human BNP promoter and provide data suggesting that binding of HIF-1α/VP16 and an unknown E-box binding factor are critical to up-regulation of *BNP*. Understanding the mechanism by which HIF-1α regulates *BNP* will add to our understanding of cardiac pathophysiology and stress response in disease. Furthermore, because BNP is protective in a number of scenarios, these results provide further rationale for the therapeutic use of HIF-1α/VP16 in ischemic disease. Thus, constitutive activation of HIF-1α (HIF-1α/VP16) may be therapeutic in ischemic disease as a result of up-regulation of HIF-1α target genes, including *BNP*, angiogenic genes (*VEGF* and growth factors), stress-induced genes (*inducible nitric-oxide synthase*), and others. However, there is still a great need to investigate the detailed effects of HIF-1α/VP16 in vivo in ischemic disease. Comprehensive gene expression profiling coupled with functional genomic studies should elucidate the detailed mechanisms of HIF-1α action and will validate or alleviate concerns that HIF-1α activation might have adverse effects or contribute to other disease states.

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