HYPOXIA-INDUCED REGULATION OF mRNA STABILITY

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Abstract: Because molecular oxygen is essential for generating cellular energy in aerobic organisms, and because survival depends on this fundamental requirement for oxygen, all higher organisms have evolved numerous diversely regulated mechanisms to detect and respond to potentially life-threatening occurrences of decreased oxygen availability (hypoxia). While the oxygen-dependent regulation of gene expression involves both transcriptional- and post-transcriptional mechanisms, investigations have focused mainly on mechanisms working at the transcriptional level. In this review, the focus is on a growing body of work that looks at post-transcriptional mechanisms acting at a level of mRNA stability.

1. INTRODUCTION

The adaptation of mammalian organisms to hypoxia involves a complex system of short-term, intermediate, and long-term actions. In the short run, the organism's ventilation rate increases to supply more oxygen, while over a period of days, the production of red blood cells rises to boost the oxygen-carrying capacity of the blood. Finally, long-term angiogenesis enhances the organism's vasculature, permanently improving its ability to survive in a hypoxic environment. All three responses are driven by an increased activity of hypoxia-sensitive genes, as are most of the known adaptations to hypoxia.

Hypoxia-induced regulation of gene expression can occur at the levels of transcription, mRNA stability, or both. The mRNA stability is an important factor regulating gene expression because mRNA half-lives directly affect

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the steady-state mRNA levels, and mRNA abundance is the primary determinant of translationally derived protein quantities. Hypoxia is known to specifically increase the stability of various mRNAs such as vascular endothelial growth factor (VEGF), tyrosine hydroxylase (TH), and erythropoietin (EPO). Below, we discuss how the hypoxia-induced stabilization of these and other mRNAs comes about as a result of interactions between specific protein factors and regulatory sequences within the mRNAs.

2. VEGF mRNA STABILITY

VEGF, an important angiogenic factor, plays crucial roles during embryonic development, wound healing, and the neovascularization of oxygen deprived tissues (Folkman 1992, Carmeliet *et al* 1996, Ferrara *et al* 1996). Further, hypoxia-induced VEGF expression is a prominent pathological feature associated with solid tumor progression (Shweiki *et al.* 1992 & Plate *et al.* 1994). The mechanisms by which hypoxia induces VEGF mRNA expression is dual in nature, taking place not only at the level of gene transcription, but at the level of mRNA stability as well.

While VEGF mRNA has a half-life in the range of 40-60 mm during normoxia, several investigators have shown that hypoxia (1-2% O_2) increases it by as much as 3 to 4-fold (Ikeda *et al* 1995, Levy *et al.* 1995, 1996a, Shima *et al* 1995, Stein *et al* 1995, Dibbens *et al*. 1999). Analysis of the VEGF mRNA 3'-untranslated region (3'UTR) shows several sequence motifs that could be important in regulating VEGF mRNA stability, including nine canonical AUUUA sequences, one AUUUUA sequence, and one AUUUUUA sequence. *In vitro* RNA decay assays show that two fragments of the VEGF 3' UTR, which correspond to the NsiI-XbaI and StuI-NsiI restriction fragments, appear to be functional instability elements (Levy *et al* 1996a). Moreover, *in vitro,* the NsiI-XbaI fragment is necessary for the hypoxia-induced stabilization of VEGF mRNA. This requirement is supported by the observation that half-lives of VEGF transcripts containing the NsiI-XbaI fragment are significantly longer when incubated with S100 cytoplasmic extracts from hypoxic cells. These transcripts form hypoxia-inducible protein-RNA complexes in electromobility shift assays (EMSA). Note that transcripts lacking the previously mentioned fragment are neither stabilized by hypoxia, nor do they form EMSA complexes. Further mapping of the binding sites has revealed three similar AU-rich elements corresponding to nucleotides 1472-1510, 1508-1573, and 1631-1678 of VEGF mRNA 3'UTR region, all of which form hypoxia-inducible EMSA complexes (Levy *et al,* 1996b). Importantly these elements are conserved

between the human and rat VEGF 3' UTRs (Levy *et al* 1997). RNA affinity purification and UV light cross-linking has identified three proteins of 32, 28, and 17 kDa that participate in complex formation. One of these hypoxia-inducible protein factors was identified as a 36 kDa HuR factor (Levy *et al* 1998). HuR appears to be necessary for the post-transcriptional induction of VEGF expression by hypoxia, although hypoxia does not appear affect the total cellular concentration of HuR. A decrease in HuR accumulation, caused by overexpression of HuR antisense RNA, abolishes the hypoxic stabilization of VEGF mRNA, while the overexpression of HuR augments the hypoxic inducibility of VEGF mRNA stability (Levy *et al* 1998).

Another AU-rich element (125 bases long) has been identified in the proximal region of the 3' UTR of the human VEGF mRNA (Claffey *et al.* 1998). Studies of function show that the presence of this element in the chimeric reporter constructs results in significantly higher hypoxia-induced activity of the reporter construct than the construct without the VEGF insert. This element forms hypoxia-inducible complexes with cytoplasmic protein extracts. The UV light cross-linking studies of the hypoxia inducible complexes identified several hypoxia-induced proteins of 90/88, 72, 56, and 46 kDa. A later study shows that one of the proteins involved in forming the hypoxia-inducible complex was heterogeneous nuclear ribonucleoprotein L (hnRNPL) (Shih & Claffey 1999). This protein binds to the VEGF mRNA *in vivo.* Blocking the VEGF mRNA interaction with hnRNPL using an antisense oligonucleotide results in the destabilization of VEGF mRNA (Shih & Claffey 1999).

In spite of the presence of the hypoxia-inducible protein-binding elements within the 3' UTR of VEGF mRNA, it is clear that the 3' UTR alone is not sufficient to confer hypoxic-inducibility to the chimeric mRNA. Interestingly, a recent study shows that recapitulation of the hypoxic induction of VEGF mRNA stability requires elements located in the 5' UTR and 3' UTR, as well as the coding region of VEGF mRNA (Dibbens *et al* 1999). This study also shows that the destabilizing elements are located in the 3'UTR and 5'UTR, as well as in the coding region of the VEGF mRNA. Either of these three regions can independently promote mRNA degradation and act in an additive fashion to promote rapid degradation under normoxic conditions (Dibbens *et al.* 1999).

The von Hippel-Lindau tumor suppressor protein (pVHL) modulates the regulation of VEGF mRNA stability (Levy *et al* 1996b). Mutations and the loss of heterozygosity for pVHL are associated with hereditary VHL disease, which results in hemangioblastomas, renal clear cell carcinomas, and pheochromocytomas (Latif *et al.* 1993). These tumors are highly vascularized, and in fact, hemangioblastomas and angiomas are actually formed of capillaries—an indication of an active angiogenic process (Plate *et*

al. 1994). VHL-associated hemangioblastomas have high levels of VEGF mRNA in stromal cells, while renal tumors show an increase in the expression of VEGF in clear cells (Stratmann *et al.* 1997). Interestingly, VHL patients show higher levels of VEGF in the aqueous fluid of the anterior chamber of the eye, as well as in serum, when compared to a control group—even in cases without clinically present tumors (Los *et al.* 1997). This finding suggests that an increase in VEGF production, which results from either abnormal- or nonexistent activity of the VHL protein, could be an early factor in the development of a VHL phenotype preceding tumor growth.

Cell culture studies show an increase in the expression of VEGF mRNA in renal carcinoma cells (RCCs) that lack the normal VHL gene. Furthermore, in these cells, the expression of VEGF cannot be further stimulated by hypoxia. Reintroduction of the wild-type VHL represses VEGF mRNA, and it reestablishes the hypoxia inducibility (Iliopolous *et al.* 1996, Siemeister *et al.* 1996). Results are similar for a platelet-derived growth factor (PDGF) B chain and a glucose transporter, Glut-1 (Iliopoulos *et al.* 1996). Two laboratories using two different RCC cell lines have reported no effect of VHL on VEGF promoter activity and no change in transcription rates measured by nuclear runoff assays (Gnarra *et al.* 1996 & Iliopoulos *et al.* 1996). In contrast, measurements of the VEGF mRNA $t_{1/2}$ showed a decreased half-life in the presence of wild-type VHL (Ilipoulos *et al.* 1996). A corresponding change in the formation of a VEGF mRNA-protein complex has also been reported (Levy *et al.* 1996b). The identity of the protein factor forming this VEGF mRNA associated complex, however, is unknown, and the mechanism by which VHL regulates mRNA stability remains unclear. The mechanism is attractive, however, because more VHL is present in the cytoplasm than in the nucleus, indicating functionality in the cytoplasm. While most studies of VEGF mRNA stability have used actinomycin D both as an inhibitor of transcription and to measure the mRNA halt-life, recent findings show that actinomycin D causes VHL to translocate to the nucleus (Lee *et al.* 1999). Therefore, the measured decrease in the VEGF mRNA stability in the presence of wild-type VHL could be secondary to the effect of actinomycin D, which results from a decrease in the concentration of cytoplasmic VHL.

3. TYROSINE HYDROXYLASE mRNA STABILITY

Tyrosine hydroxylase (TH), the rate limiting enzyme in the biosynthesis of catecholamines, is expressed in specific populations of neurons in the central and peripheral nervous systems, in the neuroendocrine cells of

adrenal medulla and carotid body, and in cultured cell lines such as the pheochromocytoma-derived PC 12 cell line. As discussed below, evidence is growing that the regulation of TH gene expression involves changes in TH mRNA stability, as well as transcriptional control.

TH mRNA is a stable message with a half-life that varies from 9 to 16 h in various subclones of PC12 cells (Summerhill *et al.* 1987, Fossom *et al* 1992, Vyas *et al.* 1992). It is enhanced during the differentiation of neuroblastoma cells (Summerhill *et al* 1987), and during the stimulation of the protein kinase C pathway in PC 12 cells (Vyas *et al.* 1990). In contrast, the stability of TH mRNA does not change in PC 12 cells during the stimulation of TH mRNA expression by dexamethasone or forskolin (Fossom *et al.* 1992). A recent study demonstrates that substantial differences exist in basal TH mRNA turnover rates between different neuronal populations, ranging from as short as 6-7 h in the dopaminergic neurons of the arcuate nucleus to as long as 11-23 h in the dopaminergic midhypothalamic neurons (Maurer *et al.* 1997).

The stability of TH mRNA is also regulated by oxygen tension. Expression of the TH gene is induced by hypoxia at the level of transcription and at the level of RNA stability in PC 12 cells (Czyzyk-Krzeska *et al* 1994a). The initial increase in TH mRNA is caused primarily by fast, transcriptional induction. The transcriptional rate, however, declines during sustained hypoxia, and augmented mRNA stability takes over as the primary contributor to the accumulation of TH mRNA. Hence, an increase in TH mRNA stability is necessary to maintain TH mRNA levels at the elevated concentration during long-term hypoxia.

A hypoxia-inducible protein-binding sequence (or HIPBS) has been identified that is a 27-base-long pyrimidine-rich sequence within the TH mRNA 3'UTR (1552-1578 bases of TH mRNA). The HIPBS binds the protein factors in a hypoxia-inducible manner in PC 12 cells (Czyzyk-Krzeska *et al.* 1994b, Czyzyk-Krzeska and Beresh 1996), catecholaminergic cells of the superior cervical ganglia, and in the dopaminergic cells of the carotid body (Czyzyk-Krzeska *et al.* 1997). Mutational analysis reveals that the optimal protein-binding site is represented by the motif (U/C)(C/U)CCCU within the pyrimidine-rich sequence, where the underlined cytosines represent the core binding site (Czyzyk-Krzeska and Beresh 1996). This motif is conserved in TH mRNAs of different species, which implies that the formation of the ribonucleoprotein complex associated with the HIPBS is involved in the physiological regulation of TH mRNA stability in catecholaminergic cells.

The HIPBS is definitely a stabilizing element that is required for both constitutive and hypoxia-regulated control of TH mRNA (Paulding *et al.* 1999). Importantly, while the HIPBS alone confers a constitutive increase in

mRNA stability to a chloramphenicol acetyltransferase (CAT) reporter mRNA, additional elements located in the coding region of the gene appear to be necessary for hypoxic regulation. The HIPBS-binding protein is represented by two isoforms of a 40 kDa poly(C) binding protein (PCBP), also known as αCP or hnRNPE (Kiledjian *et al.* 1995, Wang *et al.* 1995, Holcik & Liebhaber 1997, Gamarnik & Andino 1997). Expression of the $PCBP_1$ isoform is induced by hypoxia in PC12 cells.

4. EPO mRNA

Erythropoietin (EPO) is a glycoprotein hormone essential for regulating red blood cell production during environmental hypoxia. Hypoxia induces EPO gene expression, increasing mRNA levels up to 100-fold and serum levels of EPO protein up to 1000-fold. Most studies investigating the mechanisms regulating EPO gene expression by hypoxia were performed in two cells lines derived from liver tumor, HepG2 and Hep3B. The major mechanism responsible for the hypoxic induction of EPO gene expression is transcription, although here too, evidence suggests a role for the hypoxic stabilization of EPO mRNA. An accurate determination of the half-life of EPO mRNA, however, is complicated, because the stability of the EPO message is modulated by ongoing transcription and translation (Goldberg *et al.* 1991).

Analysis of the 3' untranslated region (3' UTR) of EPO mRNA confirms the putative stability and instability elements. Deletion of the 186-base conserved sequence from the distal 3' UTR of EPO mRNA increased the $t_{1/2}$ from 2 h to 15 h (Ho *et al.* 1995). This finding suggests that the deleted fragment of the 3' UTR could contain an RNA instability element, while the remaining region, which is 5' from the deleted region, could contain an RNA stability element.

Deletion of the 104 bases immediately downstream from the EPO translation stop codon results in a destabilization of EPO mRNA from 7 h to 2.6 h, thus indicating that this fragment could contain an RNA stability element (McGary *et al.* 1997). These experiments, however, were performed in the presence of actinomycin D, which stabilizes EPO mRNA in a nonspecific manner. Thus, it is difficult to estimate the role of this fragment of the EPO 3'UTR in regulating EPO mRNA stability. This putative EPO mRNA stability region was previously found to bind cytoplasmic proteins (Rondon *et al.* 1991, 1995). UV light cross-linking and SDS-PAGE analysis of the EPO RNA-protein complexes revealed two bands of 70 and 135-140 kDa (Rondon *et al.* 1991). Except in the brain and the spleen, however, the binding activity in most cell lines and tissues studied was not induced by

hypoxia. The binding of these proteins to EPO mRNA was redox-sensitive and required the use of reduced thiol groups (Rondon *et al.* 1995).

The sequence of the protein-binding putative EPO mRNA stability element shows a high level of homology with the HIPBS stability element identified within the TH mRNA. This homology led to the identification of two 40kDa poly(C) binding proteins, ($PCBP_1$ and $PCBP_2$), as part of the EPO mRNA-associated complex (Czyzyk-Krzeska & Bendixen, 1999). In contrast to PCl2 cells, however, the expression of the PCBP1 is not regulated by hypoxia in Hep3B or HepG2 cells, implying that they might participate in a constitutive- rather than hypoxia-inducible stability of EPO mRNA.

5. MISCELLANEOUS GENES AND mRNA STABILITY

The hypoxia-induced stabilization of few additional genes has been described but in less detail than for the above genes. The additional genes include the glucose transporter 1 (GLUT1), which is a mammalian specific carrier facilitating glucose uptake in brain cells (Bruckner *et al.* 1999), and a plasminogen activator inhibitor-1 (PAI-1), which is a serine protease inhibitor that profoundly inhibits fibrinolysis. As such, PAI-1 may have important implications for the pathogenesis of diseases associated with hypoxemia (Pinsky *et al.* 1998). Both of these messages are stabilized by hypoxia, but the mechanisms mediating stabilization remain unknown.

Hypoxia was recently shown to differentially regulate the expression of the iron-regulatory proteins 1 and 2 (IRP1 and IRP2) (Hanson *et al.* 1999). These proteins are RNA binding proteins that post-transcriptionally regulate the expression of mRNAs that code for proteins involved in the maintenance of iron and energy homeostasis. IRP1 and IRP2 bind specific RNA stem-loop structures termed iron-responsive elements (IREs) that may be located in both the 5'- and 3' UTRs of specific mRNAs. Examples of IRE containing proteins include, ferritin (iron storage), transferrin receptor (iron transport), erythroid-aminolevulinate synthase (heme biosynthesis), and mitochondrial aconitase (energy metabolism). With respect to the stem-loop structures, the control of IRP1 and IRP2 regulation for given transcripts is functionally quite similar to the mechanisms discussed above for TH, VEGF, and EPO.

It should be mentioned that hypoxia has been shown to decrease mRNA stability for the following three genes: endothelial constitutive nitric oxide synthase (eNOS) (McQuillan *et al.* 1995), mitochondrial manganese-containing superoxide dismutase (Mn-SOD), and the cytosolic copper and zinc-containing superoxide dismutase (Cu,Zn-SOD) (Jackson *et al.* 1996).

Again, the mechanisms mediating the effects of hypoxia in these cases remain unknown.

6. CONCLUSION

Since the abundance of mRNA is an important factor in establishing cellular protein levels, control of the transcript half-life is vitally important in determining an organism's ability to respond to life-threatening environmental changes. For this reason, the role oxygen plays in regulating mRNA stability and, ultimately, gene expression is deservedly gaining increased attention. While common post-transcriptional regulatory mechanisms for many genes are currently emerging in the form of closely related cis-elements and even shared trans-acting factors, the complete elucidation of regulatory mechanisms remains quite complicated. Considering the physiological importance of hypoxia on the best-characterized genes, VEGF, TH, and EPO, it is obvious that a thorough understanding of the hypoxia-induced processes that control mRNA stability is clinically significant, offering much potential for pharmacological interventions.

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