

Novel Function of E3 Ubiquitin Ligase Siah2 to Regulate ROS Metabolism

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ABSTRACT

The seven in absentia homolog (Siah) family proteins are components of E3 ubiquitin ligase complexes that catalyze the ubiquitination of proteins. Siah proteins target their substrates for proteasomal degradation. Several studies have reported that Siah proteins are involved in tumorigenesis and angiogenesis, particularly through the Ras and HIF-1 α signaling pathways in hypoxic response. Recent studies also have reported that Siah2 stabilization impairs the NF-E2-related factor 2 (Nrf2) signaling pathway, which is a master regulator of reactive oxygen species (ROS) metabolism. Hypoxia induces the phosphorylation of Nrf2 and then decreases the Keap1-mediated degradation of Nrf2 compared with that under normoxia. In addition, hypoxia-induced Siah2 provides a dominating Nrf2 degradation pathway over that of Keap1 under hypoxia. Given their critical roles in ROS metabolism, Siah proteins are attractive targets for effective therapy under particular conditions such as hypoxia and hypoglycemia.

Key words: Siah2; NF-E2-related factor 2; Reactive oxygen species; Hypoxia; Ischemia reperfusion

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INTRODUCTION

Ischemia-reperfusion injury occurs when blood flow is disrupted and subsequently reintroduced. The rapid reintroduction of oxygen generates reactive oxygen species (ROS), leading to oxidative damage^[1]. Oxidative damage is implicated in pathophysiological processes, including myocardial infarction and brain stroke^[2]. To prevent oxidative damage to tissues, we must understand the molecular mechanisms underlying ROS generation and degradation during ischemia and reperfusion. ROS generation during ischemia and reperfusion results from a sequence of three steps. In the first step, ischemia occurs, which involves insufficient blood flow to provide adequate oxygenation, leading to tissues being exposed to hypoxia. Hypoxia induces and activates ROS generators, such as NADPH oxidases and xanthine oxygenase. In the second step, the restoration of blood flow reintroduces oxygen into the affected area. In the third step, this re-oxygenation results in ROS generation as a natural byproduct of the normal metabolism of oxygen. Hypoxic ischemia causes the excessive generation of ROS^[3]. In contrast, extra ROS accumulation is brought back to normal by the induction of antioxidant proteins. The levels of these proteins are increased by activation of their production at the transcriptional level in response to oxidative stress^[4]. Antioxidant responsive elements AREs are cis-regulatory elements that are crucial for the expression of cytoprotective genes. Nrf2 is known to be a key transcriptional activator of AREs. Under normal conditions, Nrf2 is constantly degraded through the ubiquitin-proteasome pathway in a Keap1-mediated manner. Keap1 maintains steady-state levels of Nrf2 and blocks Nrf2-mediated transcription^[5]. Moreover, under oxidative stress, oxidized substance

modify Keap1, leading to a conformational change in Keap1 and/or stress-mediated kinases modify Nrf2, leading to a conformational change in Nrf2^[6]. Either or both of these mechanisms allow the liberation of Nrf2 from Keap1, leading to the activation of AREs. In this manner, the regulation of Nrf2 under oxidative conditions is well characterized. However, despite the involvement of ROS during hypoxia in ischemia-reperfusion injury, the regulation of Nrf2 under hypoxic conditions has remained unclear. Hypoxia induces the phosphorylation of Nrf2; then Keap1-mediated degradation of Nrf2 is decreased compared with that under normoxia. In addition, hypoxia-induced Siah2 provides a dominating of Nrf2 degradation pathway stronger than over that by Keap1 under hypoxia^[7]. These phenomena suggest that Siah2 is involved in ROS metabolism. Therefore, in that case, Siah proteins would be attractive targets for effective therapy under particular conditions such as ischemia-reperfusion injury.

IDENTIFICATION OF SINA AND SIAH FAMILY

The seven in absentia (Sina) gene was originally identified in *Drosophila*^[8]. Murine and human homologs of the Sina gene have also been identified. In mice, Sina homologs (Siah) were isolated as three Siah proteins, Siah1a, Siah1b, and Siah2, which are encoded by different genes^[9]. In contrast, the human Siah proteins consist of two homologs, Siah1 and Siah2, which are encoded by the *SLAH1* and *SLAH2* genes, respectively, and apparently have distinct but overlapping functions^[10].

FUNCTION OF SINA AND SIAH PROTEINS

The Sina protein is required for regulation of the R7 signaling pathway in photoreceptor cell development of *Drosophila*. The loss of functional Sina protein causes a deficiency of R7 precursor cells^[11]. Sina binds to the Sevenless pathway protein phyllopod (PHYL) and Ebi, and an ubiquitin ligase complex consisting of them target a transcriptional repressor, Tramtrack, for ubiquitin-dependent proteolysis, resulting in a cell-fate decision in the development of *Drosophila eye*^[12].

In *Xenopus*, Siah2 is expressed maternally and is later restricted to the brain, spinal cord and developing and mature eyes. Siah2 overexpression during *Xenopus* development causes the small-eyes phenotype^[13].

On the other hand, in mammals, a similar ubiquitin ligase complex is formed and functions in ubiquitin-dependent proteolysis by the well-described anaphase-promoting complex (APC), Skp1/Cullin/F-box complex (SCF), and Siah/Ebi/Siah interacting protein (SIP) complex^[14,15]. Siah contains functional binding sites that recognize a peptide motif within many substrates and adaptors^[16]. Siah proteins bind to a highly conserved PxAxVxP motif in their substrates^[17], and interact with numerous cellular proteins such as a transcriptional repressor, Tramtrack; peptide inhibitor, PHYL; the oncogenic protein, β -catenin; a nuclear receptor co-repressor, N-CoR; a motor protein Kid; and the tumor suppressor TGF- β induced early gene (TIEG)^[12,15,18-20]. In some cases, such interaction requires an adaptor protein such as PHYL, and SIP to degrade *Drosophila* Tramtrack and mammalian β -catenin, respectively.

Siah proteins are potent RING finger E3 ubiquitin ligases that catalyze the ubiquitination of proteins, bind to E2 enzymes, and target their substrates for ubiquitin degradation. Siah2 is a RING finger type ubiquitin ligase with a catalytic RING domain on its N-terminus, followed by two zinc fingers and a C-terminal substrate

binding domain (SBD)^[16]. Siah2 limits its own availability through self-ubiquitination^[21] and is a known regulator of hypoxia-activated signaling pathways^[22].

UPSTREAM SIGNALING PATHWAYS OF SIAH PROTEINS

Siah1 expression is enhanced by p53, which is activated by DNA damage. Expressed Siah1 is phosphorylated by ATM/ATR after DNA damage^[23]. The tumor suppressor HIPK2, which phosphorylates p53 at Ser 46^[24], contributing to p53-mediated apoptosis induction, is a substrate of Siah proteins. The phosphorylation of Siah1 by ATM/ATR disrupts the interaction of HIPK2 and Siah1, thereby stabilizing HIPK2, resulting in the promotion of apoptosis induction.

Treatment with a chemical inhibitor against Phosphoinositide 3-kinase (PI3K) decreases Akt activity and the expression levels of hypoxia-induced *Siah2* mRNA. The expression of Siah2 is induced when an active form of Akt is introduced into cells^[25]. Therefore, the Akt-dependent pathway may be one of the critical signaling pathways to induce Siah2, although the transcription factor that induces *Siah2* mRNA is still unknown.

Siah2 is subjected to phosphorylation, which increases its activity under hypoxia. This phosphorylation of Siah2 is inhibited when cells are exposed to a chemical inhibitor against p38 mitogen-activated protein kinase (MAPK). Siah2 contains the SQ/TQ amino acid sequence motif, a conserved motif for p38 MAPK phosphorylation. S29 and T24 are major phosphorylation sites of Siah2. The phosphorylation of Siah2 by p38 MAPK results in stabilization and subsequent activation of the Siah2 protein in the cytoplasm^[26].

In addition, the expression of Siah2 is up-regulated by estrogen in estrogen-receptor (ER)-positive breast cancer cells, in which Siah2 degrades the repressor of ER signaling, N-CoR, resulting in resistance to apoptosis induction and affecting mitochondrial function^[18]. Furthermore, ubiquitin specific peptidase 13 (USP13) reduces the substrate degradation activity of Siah2 protein with a concomitant inhibitory effect on activity of Siah2 under normoxia^[27].

FUNCTION OF SIAH AS A TUMOR SUPPRESSOR PROTEIN AND ITS ROLE IN CANCER PROGRESSION

Increasing evidence indicates that Siah has an important role in tumor development and progression. Previous studies suggested the tumor suppressive role of Siah, particularly Siah1. The function of Siah in cancer progression was first investigated in pancreatic cancer. In human pancreatic cancer cells, overexpression of both mutated Siah1 and Siah2 was found to cause growth arrest^[28]. In addition, overexpression of Siah1 arrested the growth of hepatoma cells^[29]. Furthermore, the expression of Siah1 transfected into U937 lymphoma cell suppressed the tumorigenesis of these cells when injected into mice^[30]. Finally, in other cancers, such as liver and gastric cancers, low levels of Siah1 expression promoted cancer progression^[31].

A dominant negative Siah protein was also found to inhibit ERK signaling and cell proliferation, increase apoptosis induction, and reduce tumorigenesis of A549 human lung cancer cells, when injected into nude mice^[32]. In addition, inhibition of the expression of Siah had an anti-tumorigenic effect in mouse melanoma, SW1 cells,

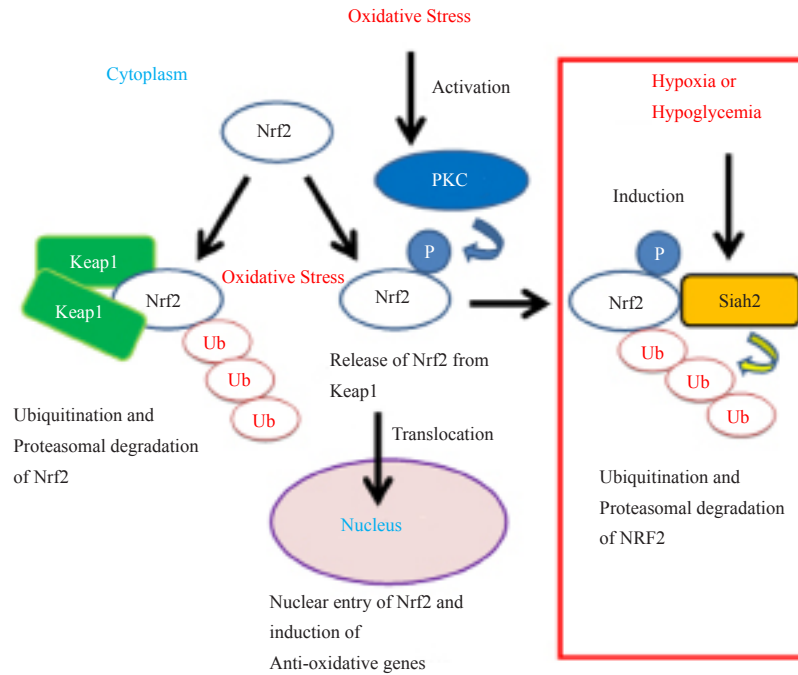


Figure 1 Under basal conditions, Nrf2 is degraded in Keap1-dependent manner. Under oxidative stress, PKC is activated and phosphorylates Nrf2, leading to nuclear entry of Nrf2. In other hands, such stress as hypoxia or hypoglycemia induces the phosphorylation of Nrf2 and thus decreases Keap1-mediated degradation of Nrf2 compared with that under basal conditions. Especially, concomitant stabilization of Siah2 provides a dominating degradation of Nrf2 stronger than that by Keap1 under basal conditions.

and breast cancer cells^[33]. Inhibition of the binding of Siah with the substrates through PHYL also reduced tumor growth, angiogenesis, and metastatic spread by the inactivated hypoxic response pathway^[34]. Furthermore, *in vivo*, a chemical inhibitor of Siah2, menadione, abolished the growth of xenograft tumors in nude mice^[35]. The genetic knockout of Siah proteins is valuable in establishing the significance of biochemical and cell biological studies. Finally, in both Siah1a and Siah2 null mice, tumor progression to an advanced stage was inhibited and metastasis was reduced^[36]. These findings suggest that the inhibition of Siah expression is effective for attenuating tumor growth.

FUNCTION OF SIAH IN HYPOXIC RESPONSE AND ITS ROLE IN MYOCARDIAL ISCHEMIA

Hypoxia-inducible factor-1 (HIF-1) is a master transcriptional factor that plays a critical role in protecting cells against hypoxic stress within the range of physiological normoxia to mild hypoxia^[37]. Under normal conditions, HIF-1 expression is regulated by the hydroxylation of its proline residues^[38]. Siah2 protein targets prolyl hydroxylases (PHD) 3 and 1 that are responsible for this function for degradation, concomitantly facilitating HIF-1 stabilization under an oxygen concentration of 2%-5%^[39]. The loss of Siah2 attenuates HIF-1 protein expression levels, leading to decreased proliferation, and increased apoptosis induction in cancer cells. In Siah2 knockout mice, tumor onset was found to be delayed; in addition, the mice exhibited an abrogated response to hypoxic conditions. At cellular and tissue levels, the expression of Siah2 mutants under hypoxic conditions led to significantly lower protein levels of HIF-1, resulting in reduced hypoxia-induced gene expression^[34].

Recent studies have also suggested that NF-E2-related factor (Nrf2), which plays an important role in ROS metabolism, is negatively regulated by Siah2 under stressful conditions^[7,40]. Under basal conditions, Nrf2 is a well-known substrate for Kelch-like ECH-associated protein 1 (Keap1)^[6]; however, Siah2, but not Keap1, is involved in the degradation of Nrf2 under particular conditions. For example, under hypoxia, Nrf2 is phosphorylated by protein kinase C (PKC), which triggers the release from Keap1. Knockdown of Siah2 rescues hypoxia-induced reduction of both Nrf2 mutants mimicking phosphorylation at Ser40 and lacking this phosphorylation site, suggesting that Siah2 contributes to the degradation of Nrf2 irrespective of its phosphorylation status^[7] (Figure 1). In addition, the knockdown of Siah2, but not Keap1, was shown to significantly attenuate the hypoglycemia-mediated reduction of Nrf2 expression^[40].

Mitochondria are critical for cell survival, and their morphology is associated with susceptibility to cell death signals^[41]. In ischemia-induced cardiomyocyte cell death, Siah2 regulates the availability of the mitochondrial scaffolding protein AKAP121 and subsequent mitochondrial dynamics. Reduced availability of AKAP121 due to Siah2 relieves Drp1 inhibition by PKA and increases its interaction with Fis1, resulting in mitochondrial fission. In cells lacking Siah2, the level of this fission is decreased and ischemia-induced apoptosis of cardiomyocytes is reduced.

RNAi inhibition of both Siah1 and Siah2 reduces infarct size while improving cardiac function after ischemia-reperfusion injury of hearts. Moreover, *in vivo* RNAi inhibition of both Siah1 and Siah2 was found to augment the cardioprotective function of netrin-1^[42].

FUNCTION OF SIAH IN ROS METABOLISM

The major sources of ROS, including mitochondria, NADPH oxidases, xanthine oxyganase, and Ca²⁺ flux, are activated

during ischemic hypoxia, representing important cytotoxic mechanisms^[3]. ROS causes lipid peroxidation as well as protein and DNA oxidation, and it also stimulates ischemic cells to secrete inflammatory cytokines and chemokines that induce cell damage. Cellular oxidative stress defense systems are tightly regulated through the synthesis and degradation of many transcriptional factors such as Nrf2^[5]. Ischemia reperfusion generates ROS and leads to cellular damage and oxidative stress in hypoxia and subsequent reoxygenation.

The role of Siah1/2 in ROS production and degradation remains unclear; however, some studies have suggested that inhibition of the expression and function of Siah might be an attractive approach to impair ROS metabolism. Interfering with Nrf2 down-regulation through Siah inhibition is effective to induce the expression of cytoprotective genes^[7,40], as it may have an impact on the cellular adaptive responses to oxidative stress more broadly than the inhibition of Keap1 alone.

CONCLUSION

Siah2 protein is a regulator of several signaling pathways such as ER, HIF-1 α , and Ras. Recent work has demonstrated that Siah2 protein is a promising therapeutic target in pathological conditions accompanied by a low level of Nrf2 expression^[7,40]. The inhibition of Siah2 expression restores the decreased expression of Nrf2-inducible genes and subsequently improves symptoms. Past data focuses on the inhibition of Siah2 expression in cell lines alone. The effect of Siah2 inhibition on the Nrf2 signaling pathway remains unclear in animal models. Furthermore, the incidence of Nrf2 and Siah2 interaction *in vivo* remains unclear. Therefore, more precise investigations are needed for a understanding of the direct association of Nrf2 and Siah2 and of the molecular mechanism underlying this relationship *in vivo*.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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