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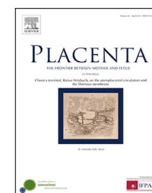

Dynamic regulation of HIF1A stability by SUMO2/3 and SENP3 in the human placenta

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A B S T R A C T

Introduction: Hypoxia-inducible factor 1A (HIF1A) stability is tightly regulated by hydroxylation and ubiquitination. Emerging evidence indicates that HIF1A is also controlled by the interplay between SUMO-specific ligases, which execute protein SUMOylation, and Sentrin/SUMO-specific proteases that de-SUMOylate target proteins. Given the significance of HIF1A in the human placenta, we investigated whether placental HIF1A is subject to SUMOylation in physiological and pathological conditions.
Methods: Placentae were obtained from normal and pregnancies complicated by preeclampsia. Human choriocarcinoma JEG3 cells were maintained at either 21% or 3% oxygen or exposed to sodium nitroprusside (SNP). Cells were transfected with SUMO2/3 constructs with and without Mg132, a proteasome inhibitor. Expression, distribution and associations of SUMO/SENPs and HIF1A were evaluated by Western blotting, immunohistochemistry and co-immunoprecipitation.
Results: HIF1A-SUMO2/3 associations peaked at 9–10 weeks, while its deSUMOylation by SENP3 was greatest at 10–12 weeks. In E-PE, HIF1A deSUMOylation by SENP3 was significantly elevated, while HIF1A-SUMO2/3 associations remained constant. *In vitro*, overexpression of SUMO2/3 de-stabilized HIF1A in hypoxia, and abrogated HIF1A expression following Mg132 treatment in normoxia. Hypoxia and SNP treatments promoted SENP3 nuclear redistribution from nucleoli to the nucleoplasm where it associates with HIF1A.
Conclusion: During early placental development, SUMOylation events control HIF1A stability in an oxygen-dependent manner. In E-PE, enhanced deSUMOylation of HIF1A by SENP3 may in part contribute to increased HIF1A activity and stability found in this pathology.
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1. Introduction

Oxygen plays a key role in a variety of physiological and pathological processes [1]. The human placenta is no exception as it exemplifies the importance of physiological oxygen changes in orchestrating trophoblast differentiation events required for shaping the development of this organ, thereby ensuring proper fetal growth [2,3]. The adaptive cellular response to hypoxia is predominantly mediated by a highly conserved hypoxia-inducible factor (HIF) family of transcriptional regulators [4]. The transcriptionally active HIF heterodimer is composed of one of oxygen responsive α-subunits (HIF1A, HIF2A and HIF3A) and the constitutively expressed β-subunit (HIFB/ARNT) [5].

In hypoxia, HIF1A stability is preserved, leading to its nuclear accumulation, where it binds to HIF1B, thereby recognizing hypoxia-response elements (HRE) present in the promoter region of hypoxia-responsive target genes [6]. In normoxia, HIF1A is hydroxylated at specific proline residues by prolyl-hydroxylase

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Abbreviations

ACOG	The American Congress of Obstetricians and Gynecologists
ACTB	Actin, beta
AMC	Age-matched control
B23	Nucleophosmin
CBP	cAMP-response element-binding protein (CREB) binding protein
CT	Cytotrophoblast
DAPI	4′,6-diamidino-2-phenylindole
EMEM	Eagle's Minimum Essential Medium
E-PE	Early-onset preeclampsia
EVT	extravillous trophoblast cells
FIH1	Factor inhibiting HIF1
GCM1	Glial cells missing homolog 1
H_2O_2	Hydrogen peroxide
HIF1A	Hypoxia-inducible factor 1 alpha
HIF1B	Hypoxia-inducible factor 1 beta
HRE	Hypoxia response element
IgG	Immunoglobulin G
JMJD6	Jumonji C domain containing protein 6
L-PE	Late-onset preeclampsia
MDM2	Mouse double minute 2 homolog
ODD	Oxygen-dependent degradation domain
PE	Preeclampsia
PML	Promyelocytic leukemia
pRB	Retinoblastoma protein
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SENP1	Sentrin-specific protease 1
SENP3	Sentrin-specific protease 3
SK	Syncytial Knots
SNP	Sodium nitroprusside
SUMO1	Small ubiquitin-like modifier 1
SUMO2/3	Small ubiquitin-like modifier 2/3
ST	Syncytiotrophoblast
TC	Term control
VHL	von Hippel Lindau tumour suppressor

enzymes [7] and then targeted for proteasomal degradation by the von Hippel-Lindau (VHL) protein [8]. Furthermore, Factor Inhibiting HIF1 (FIH1) negatively regulates HIF1A transcriptional activity in normoxia by hydroxylating HIF1A thereby preventing binding to its transcriptional co-activators, p300/Creb-binding protein (CBP) [9].

Emerging evidence indicates that, in hypoxic conditions, HIF1A stability is also controlled by means of SUMOylation [10,11]. SUMOylation is a post-translational protein modification event catalyzed by small ubiquitin-like modifier (SUMO) proteins that can be efficiently reversed by Sentrin/SUMO specific proteases (SENPs). In humans, four SUMO proteins (SUMO1-4) and six SENPs (SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7) have been identified, each with unique cellular localization and target specificity [12]. Using renal cancer cells it has been shown that, in hypoxia, VHL is capable of targeting SUMOylated HIF1A for degradation [10]. Furthermore, H_2O_2-induced stress prompts p300 deSUMOylation by SENP3, thereby triggering HIF1A activity in HeLa cells [13]. Thus, SUMOylation events are critical in the cellular adaptation to hypoxia [14,15] by contributing to the maintenance of HIF1A homeostasis.

We, and others, have addressed the significance of HIF1A in placental development and have reported on the mechanisms involved in the regulation of its stability and activity [16–18]. It is accepted that placental hypoxia and oxidative stress underlie the genesis of preeclampsia, a serious pregnancy disorder, and we have shown that high levels of HIF1A in preeclampsia are in part due to altered HIF1A hydroxylation [18]. However, no information is available on HIF1A SUMOylation in the human placenta. Hence, as SUMOylation processes are tightly regulated by hypoxia and oxidative stress, we sought to examine the involvement of the SUMO/SENP system in regulating HIF1A stability in the developing and preeclamptic placenta.

2. Materials and methods

2.1. Tissue collection

Placental tissues were collected after informed consent in agreement with the ethics guidelines of the Faculty of Medicine, University of Toronto and Mount Sinai Hospital, Toronto. Placentae from early gestation (7–17 weeks, n = 32) were collected from elective termination of pregnancies. Severe early-onset preeclamptic (E-PE n = 35), late-onset preeclamptic (L-PE, n = 5), normotensive preterm age-matched control (AMC, n = 29) and term control (TC, n = 5) samples were obtained immediately after delivery. Patient clinical data are summarized in Table 1. Preeclampsia was diagnosed according to ACOG criteria [19].

2.2. Choriocarcinoma cell culture

Human choriocarcinoma JEG3 cells were seeded into 6-well plates (2×10^5 cells per well) and cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA, USA) supplemented with 2 mM L-glutamine and 5% FBS. JEG3 cells were maintained at either ambient (21%) O_2 or 3% O_2 balanced with 92% N_2/5% CO_2. In parallel experiments, cells cultured at 21% O_2 were treated for 24 h with the nitric oxide donor sodium nitroprusside (SNP, 2.5–5 mM) (Sigma-Aldrich, St. Louis, MO, USA). In separate experiments, JEG3 cells were treated with the SUMOylation inhibitor ginkgolic acid (100 μM) for 24 h [20]. Cell lysates were collected in RIPA buffer for Western blot analysis.

2.3. Western blot analysis

Western blot analyses were performed as described previously [21]. Primary antibodies included mouse monoclonal anti-human HIF1A (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-$HIF1A_{p402}$ and anti-$HIF1A_{p564}$ [18], mouse monoclonal anti-human SENP1 (C-12, 1:1500; Santa Cruz Biotechnology), mouse monoclonal anti-human SUMO1 (D-11, 1:500; Santa Cruz Biotechnology), rabbit monoclonal anti-human SUMO2/3 (1:1000; Cell Signaling Technology, Beverly, MA, USA) rabbit monoclonal anti-human SENP3 (1:1000; Cell Signaling) rabbit polyclonal B23 (1:500; Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies were used for chemiluminescence detection. Secondary antibodies included goat anti-mouse IgG (1: 2000, Santa Cruz Biotechnology) and goat anti-rabbit IgG (1:2000, Santa Cruz Biotechnology). All blots were

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