Hypoxic Stress Induces Dimethylated Histone H3 Lysine 9 through Histone Methyltransferase G9a in Mammalian Cells

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Abstract

Dimethylated histone H3 lysine 9 (H3K9me2) is a critical epigenetic mark for gene repression and silencing and plays an essential role in embryogenesis and carcinogenesis. Here, we investigated the effects of hypoxic stress on H3K9me2 at both global and gene-specific level. We found that hypoxia increased global H3K9me2 in several mammalian cell lines. This hypoxia-induced H3K9me2 was temporally correlated with an increase in histone methyltransferase G9a protein and enzyme activity. The increase in H3K9me2 was significantly mitigated in G9a−/− mouse embryonic stem cells following hypoxia challenge, indicating that G9a was involved in the hypoxia-induced H3K9me2. In addition to the activation of G9a, our results also indicated that hypoxia increased H3K9me2 by inhibiting H3K9 demethylation processes. Hypoxic mimetics, such as deferoxamine and dimethyloxaloylglycine, were also found to increase H3K9me2 as well as G9a protein and activity. Finally, hypoxia increased H3K9me2 in the promoter regions of the Mlh1 and Dhhfr genes, and these increases temporally correlated with the repression of these genes. Collectively, these results indicate that G9a plays an important role in the hypoxia-induced H3K9me2, which would inhibit the expression of several genes that would likely lead to solid tumor progression. (Cancer Res 2006; 66(18): 9009-16)

Introduction

Post-translational modifications of histone NH2-terminal tails, such as acetylation, methylation, ubiquitination, and phosphorylation, are important for chromatin organization and gene transcription (1). To date, histone acetylation and methylation are among the best-characterized modifications. Histone lysine acetylations are generally associated with gene activation, whereas lysine methylation may have either positive or negative effects on transcription depending on the sites (2). For instance, methylations on histone H3 lysine 4 (H3K4), histone H3 lysine 36 (H3K36), and histone H3 lysine 79 are often associated with transcriptional activation and elongation, whereas methylations on histone H3 lysine 9 (H3K9) and histone H3 lysine 27 correlate with transcriptional repression (2). Adding to this complexity is the fact that lysine residues on histone can be monomethylated, dimethylated, or trimethylated. Trimethylated H3K9 (H3K9me3) is typically associated with constitutive heterochromatin, whereas monomethylated H3K9 (H3K9me1) and dimethylated H3K9 (H3K9me2) are mainly found in euchromatin and are associated with repressed promoter regions (3).

Several H3K9-specific methyltransferases have been identified and characterized, including Suv39h1, Suv39h2, G9a, GLP/EuHMtase 1, and SETDB1/ESET. Among them, G9a and GLP/EuHMtase 1 were found to be responsible for the dimethylation processes on H3K9 in vivo (4, 5). The genetic ablation of either G9a or GLP/EuHMtase 1 resulted in a striking loss of global H3K9me2 (4, 5). G9a was found to participate in gene silencing processes by interacting with several repressive transcriptional factors, including NRSF/REST, CDP/cut, PRDI-BF1/Blimp-1, and SHP (6–9). Recently, SETDB1/ESET has also been found to catalyze the formation of H3K9me2 both in vitro and in vivo (10).

Compared with the methylation processes that act on histone lysines, the demethylation processes are just being studied recently. The discovery of H3K4 demethylase, LSD1, provided the first evidence for the existence of any histone lysine demethylase (11). LSD1 is a flavin-dependent amine oxidase and removes the methyl group from monomethylated or dimethylated H3K4 by catalyzing the oxidation of amine (11). LSD1 has also been reported to demethylate H3K9me2 when associated with the androgen receptor (12). Recently, Trewick et al. (13) have proposed that the methyl groups on histone lysine could also be removed by an oxidative demethylation mechanism. As proposed, a novel class of demethyleases should possess iron-binding domains (e.g., Jmjc domains) and could catalyze the generation of highly reactive oxygen species (ROS) in the presence of iron, 2-oxoglutarate, and oxygen (13). These generated ROS attack the methyl groups on histone lysines and produce unstable intermediate oxidized products that spontaneously release formaldehyde, resulting in the removal of methyl groups from histone lysines (13). JHDM1 is the first discovered demethylase that uses this proposed oxidative demethylation process to remove the methyl groups from monomethylated and dimethylated H3K36 (14). Recently, JHDM2A and the family of JMJD2 histone demethylases (JMJD2A-D) were reported to demethylate H3K9 residues (15, 16). Similar to JHDM1, these newly identified H3K9 demethylases all possess Jmjc domains and require the presence of both iron and 2-oxoglutarate to be active. Importantly, the overexpression or knockdown of these H3K9 demethylases result in the alterations of global H3K9 methylations (15, 16). These discoveries indicate that H3K9 methylations are more dynamically involved in the regulation of chromatin functions than previously thought.

Aberrant gene silencing is a common phenomenon in many types of cancers. Hypoxia often occurs in a solid tumor because normal tissue vasculature can only support tumor growth within a diameter of ~2 mm (17). Most studies have focused on the up-regulation of gene expression during hypoxia, which are often mediated through hypoxia-inducible factor (HIF; ref. 18).
The elevation of gene expression, such as vascular endothelial growth factor and the glycolytic enzymes, functions either to increase oxygen availability or to adjust intracellular metabolism and thus improves cell survival under hypoxic stress. However, less attention has been paid to the down-regulation of gene expression during hypoxia. In this study, we report that exposure to hypoxia or hypoxia mimetics is able to increase global H3K9me2 in several mammalian cell lines. Our results indicate that these alterations of global H3K9me2 can be attributed to both an increase in G9a protein and enzymatic activity as well as an inhibition of histone demethylation processes under hypoxic conditions. In addition, an increase in H3K9me2 at the promoter regions of Mlh1 and Dhfr genes was found to correlate with the repression of these two genes during hypoxic stress, indicating that the hypoxia-induced H3K9me2 might play an important role in gene silencing during tumor progression.

Materials and Methods

Cell culture. Human lung carcinoma A549 cells were grown in Ham’s F-12K medium (Life Technologies, Inc., Grand Island, NY); human embryonic kidney (HEK) 293 cells were grown in DMEM (Life Technologies); mouse embryonic stem (MES) cell clones wild-type (WT), 69a knockout (69aΔ/−), and a derivative from 69aΔ/− cells with a stably transfected 69a expression vector (69aΔ/− + 69a ΔWT) were grown in DMEM supplemented with 1 × 10^5 units/mL murine ESGBRO (Chemicon, Temecula, CA); mouse embryonic fibroblast HIF-1α proficient (HIF-1α+/+) and deficient (HIF-1α−/−) cells were obtained from R. Johnson (University of California at San Diego, San Diego, CA; ref. 19) and grown in DMEM. All media were supplemented with 10% fetal bovine serum (Omega Scientific, Inc., Tarzana, CA) and 1% penicillin/streptomycin (Life Technologies). Cells were passaged twice to thrice each week and grown to 80% to 90% confluency before any treatment. Cells were exposed to hypoxic conditions in a chamber with a humidified gas mixture of 0.5% O2 and 99.5% N2 at 37°C. The levels of oxygen in chambers were verified using a gas monitor (SKC, Inc., Eighty Four, PA).

Chemicals and expression vectors. Dimethylsulfoxide was purchased from Frontier Scientific, Inc. (Logan, UT). All other reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified. The green fluorescent protein (GFP)-tagged human G9a expression vector, pEGFP-H9a, and its SET domain deletion mutant expression vector, pEGFP-H9α(SET), were kindly provided by M. Walsh (ML Sinai School of Medicine, New York, NY, ref. 8).

Western blotting. Histones were prepared from cells as described previously (20). Equal amounts of histones (5 µg) were separated over 15% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Roche Applied Sciences, Indianapolis, IN). Immunoblottings were done with dimethyl H3K9 (1:2,000; Upstate Biotechnology, Inc., Lake Placid, NY), monomethyl H3K9 (1:1,000; Upstate Biotechnology), trimethyl H3K9 (1:400; Abcam, Cambridge, MA), acetylated H3K9 (Ac-H3K9; 1:2,000; Upstate Biotechnology), or acetylated H4 (1:4,000; Upstate Biotechnology) antibodies. The primary antibodies were detected by chemical fluorescence following an enhanced chemiluminescence Western blotting protocol (Amersham, Piscataway, NJ). After transfer to PVDF membranes, all SDS-polyacrylamide gels were stained with Bio-safe Coomasie blue stain (Bio-Rad, Hercules, CA) to assess the loading of histones. The cell extracts for HIF-1α detection were prepared as described previously (21). The immunoblottings were done with HIF-1α antibodies (1:500) from either Transduction Laboratories (BD Biosciences, San Jose, CA) or Novus Biologicals (Littleton, CO). In order to detect G9a in nuclei, the nuclear extracts were prepared using a Celllytic NuCLEAR Extraction kit (Sigma). The nuclear extracts containing 40 µg protein per sample were separated over 7.5% SDS-polyacrylamide gels. Immunoblotting was done with G9a antibody (1:500; Upstate Biotechnology). To detect overexpressed GFP-H9a or GFP-H9α(SET) fusion proteins, the whole-cell lysates containing 75 µg protein per sample were used for Western blotting.

Results

Hypoxic stress alters global H3K9 acetylation and methylation. To examine the possible effects of hypoxia on global histone modifications, histones were extracted from lung carcinoma A549 cells at selected time intervals, and the acetylation and methylation levels on H3K9 were assessed by Western blotting. Interestingly, hypoxia was found to decrease Ac-H3K9 and methylated H3K9s were scanned and subjected to densitometric analysis by Kodak 1D 3.52 (Rochester, NY) for Macintosh, and values were normalized to that obtained in the control sample.

Transient transfection and in vitro H3K9 methyltransferase activity assay. Transient transfection was done in HEK293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Twenty-four hours after transfection, the cells were exposed to hypoxia for 6 hours or 100 µmol/L deferoxamine for 24 hours. The cell extracts were prepared either for Western blotting or for the in vitro H3K9 methyltransferase (HKMT) assay as described previously (20). The pellets were used for histone extraction as described earlier.

Northern blots. After treatments, A549 cells in each 100-mm dish were mixed with Trizol (Invitrogen) and total RNA was isolated following the manufacturer’s protocol. RNA (15 µg) was separated over 1% agarose/formaldehyde gels and transferred to BrightStar-Plus positively charged nylon membranes (Ambion, Austin, TX). The Mlh1 and Dhfr probes were amplified by PCR using primers that have been described previously (22, 23), and the 9a probes were amplified using the following set of primers: 5′-GAGGACCAAGAATGGAC-3′ (sense) and 5′-CTCTAGCTGT-CAATGTGTC-3′ (antisense). The probes were labeled with [α-32P]dCTP using a Prime-a-Gene Labeling System kit (Promega, Madison, WI). Membranes were prehybridized in a hybridization solution (Sigma) for 3 hours and then hybridized overnight with the radiolabeled probes. The membranes were washed, and their radioactivity was visualized by autoradiography. The same membranes were reprobed with β-actin probes as described previously (24).

Chromatin immunoprecipitation assays. The chromatin immunoprecipitation (ChIP) assays were done as described previously (25, 26). The following sets of primers were used for PCR amplification: Mlh1: 5′-ACGCCCTGATATTGCTGCT-3′ (sense) and 5′-GTGGAATGCCG-CAAAGAAG-3′ (antisense); Dhfr, 5′-AACTGGAGGACCAAGGGAC-3′ (sense) and 5′-ATTCTATGGGAGGTTGATGCT-3′ (antisense); and Cap36, 5′-GAGGGGGGGGGGTGTGTGTGTC-3′ (sense) and 5′-GCGGGATGTGTGACTGAC-3′ (antisense). To facilitate the detection of PCR product, [32P]dCTP (0.1 µL; Perkin-Elmer, Wellesley, MA) was added into each PCR mixture. The PCR products were separated over 5% Tris-EDTA polyacrylamide gels. The gels were dried, and the radioactivity was visualized by autoradiography.

Statistical analysis. The two-tailed Student’s t test was used to determine the significance of difference between treated samples and control. The difference was considered significant at P < 0.05.
in several other cell lines, including human osteosarcoma and HEK293 cells. Because the decrease in Ac-H3K9 was more striking and appeared at earlier time intervals after hypoxia (Fig. 1A), we investigated whether this decrease in Ac-H3K9 was required for the increase in H3K9me2. As shown in Fig. 1C, trichostatin A (TSA), a histone deacetylase inhibitor, dramatically increased acetylated histone H4 but failed to block the hypoxia-induced H3K9me2. Therefore, the increase in H3K9me2 is likely caused by an imbalance between the methylation and demethylation processes that act on H3K9 residues during hypoxic stress.

Hypoxic stress increases nuclear methyltransferase G9a protein by post-translational mechanisms. Among several identified histone methyltransferases, G9a plays a dominant role in the dimethylation of H3K9 in vivo (4). To examine the role of G9a in the hypoxia-induced H3K9me2, G9a mRNA levels were measured in A549 cells following hypoxia exposure. It was found that hypoxia did not cause any measurable changes of G9a mRNA but led to a significant decrease at 6 hours after challenge (Fig. 2A).

The effects of hypoxia on G9a protein were next examined. As shown in Fig. 2B, hypoxia increased both isoforms of G9a protein that result from alternative splicing (4, 28) in the nuclei. These increases temporally correlated with the higher levels of H3K9me2 in the same cells following hypoxic stress (Fig. 2B). Consistent with previous reports showing that the overexpressed exogenous G9a proteins were exclusively present in the nuclei (4, 28), G9a protein was not detected in the cytoplasm from either exposed or unexposed cells by Western blotting (data not shown). To examine whether hypoxia up-regulates G9a protein by increasing its protein translation, cells were pretreated with cyclohexamide (a protein synthesis inhibitor) before hypoxia challenge. As shown in Fig. 2C, cyclohexamide decreased nuclear G9a protein but did not prevent its relative increase during hypoxic stress. Collectively, these results indicated that hypoxia increased nuclear G9a protein by post-translational mechanisms.

Hypoxic stress increases G9a methyltransferase activity. To examine whether the increases in nuclear G9a protein coincided with its higher methyltransferase activity, an in vitro methyltransferase assay was done. It has been reported that, in addition to G9a, many other methyltransferases also exhibit HKMT activity in vitro (4). Therefore, GFP-tagged G9a fusion protein was overexpressed in HEK293 cells to specifically measure G9a activity. In agreement with our hypothesis that hypoxia increases G9a protein levels by post-translational mechanisms, hypoxia increased the overexpressed GFP-hG9a fusion protein levels (Fig. 3). In contrast, hypoxia decreased the overexpressed GFP-hG9aΔSET) protein levels, suggesting that the methyltransferase SET domain was essential for the increase in G9a protein during hypoxic stress (Fig. 3). As shown in Fig. 3, overexpression of GFP-hG9a fusion proteins increased H3K9me2 in transfected cells but did not further increase the hypoxia-induced H3K9me2. Overexpression of GFP-hG9aΔSET) fusion proteins, which has been reported to have dominant-negative effects against the functions of endogenous G9a (9), partially blocked the increases in H3K9me2 during hypoxic stress without changing the basal levels of H3K9me2 (Fig. 3). Afterwards, the overexpressed GFP fusion proteins were immunoprecipitated and their HKMT activity was measured by an in vitro assay. Because no methyltransferase activity was detected in the samples transfected with GFP-hG9aΔSET) vectors, the activity measured in the samples transfected with GFP-hG9a specifically
represents the HKMT activity of the overexpressed GFP-hG9a fusion proteins (Fig. 3). Consistent with the increased protein levels of GFP-hG9a, hypoxia increased its methyltransferase activity (Fig. 3). It was noticed that the increases in GFP-hG9a fusion proteins and activity seemed to be less remarkable than the increases in H3K9me2 during hypoxic stress, indicating other mechanisms likely also contribute to the hypoxia-induced H3K9me2.

**Hypoxic stress also increases global H3K9me2 levels by a G9a-independent mechanism.** To further study the roles of G9a in the hypoxia-induced H3K9me2, a MES cell clone homozygous for G9a deletion (G9a−/−) and its derivative containing a stably transfected G9a expression vector (G9a+/− + G9a WT) were used. Because the H3K9me2 levels in G9a−/− MES cells were very low compared with WT cells (4), proportionately greater amounts of histones extracted from G9a−/− cells were loaded to facilitate comparisons. Exposure to hypoxia decreased Ac-H3K9, and this decrease was not affected by the presence of a functional G9a gene (Fig. 4A). Genetic ablation of G9a significantly mitigated the hypoxia-induced H3K9me2, but increases of H3K9me2 were still observed but to a lesser degree in G9a−/− cells following exposure to hypoxia (1.8-fold increase in H3K9me2 in G9a−/− cells and 2.8-fold increases in WT and G9a+/− + G9a WT cells). These results confirm that G9a is involved in the hypoxia-induced H3K9me2 but also suggest that other mechanism(s) may contribute to the hypoxia-induced H3K9me2. These alternative mechanisms may include an activation of other H3K9 methyltransferase(s) and/or inhibition of histone demethylation.

To further investigate the G9a-independent mechanism(s) by which hypoxia increases H3K9me2, cells were incubated in a methionine-deficient medium before hypoxia challenge. Because methionine is essential for S-adenosylmethionine (SAM) synthesis and SAM has a short half-life in cells (29), the withdrawal of methionine from the culture medium should cause a lowered intracellular SAM pool and a generalized inhibition of methyl transfer reactions. As shown in Fig. 4B, incubation of cells in the methionine-deficient medium for 28 hours dramatically decreased global H3K9me2 levels, indicating that the histone methylation processes were attenuated when methionine was removed from the medium. Under methionine-deficient conditions, hypoxia challenge still elevated global H3K9me2 levels compared with untreated cells (Fig. 4B). Therefore, these results provided indirect evidence to support that, in addition to the increases in G9a protein and enzymatic activity, hypoxia also increases H3K9me2 by inhibiting histone lysine demethylation processes.

**Hypoxia mimetics increase global H3K9me2 levels as well as G9a expression and activity.** Similar to hypoxia challenge, hypoxia mimetics (deferoxamine and dimethyloxalylglycine) also increased H3K9me2 and nuclear G9a protein in A549 cells (Fig. 5A). In addition, deferoxamine increased the methyltransferase activity of overexpressed GFP-hG9a fusion proteins (Fig. 5B). Similar to the findings in Fig. 4B, deferoxamine was also able to increase the global levels of H3K9me2 under methionine-deficient conditions, suggesting that the demethylation processes for H3K9 were inhibited by deferoxamine. Because a common consequence of both deferoxamine and hypoxia challenge is the stabilization and transactivation of HIF-1α, a mouse embryonic fibroblast cell line with genetic ablation of HIF-1α (HIF-1α−/−) was used to examine the role of this transcription factor in the hypoxia-induced H3K9me2. As shown in Fig. 5D, a slightly higher basal level of H3K9me2 was observed in HIF-1α−/− cells compared with HIF-1α+/+ cells. However, hypoxia increased H3K9me2 in both cell lines to similar levels (Fig. 5D), indicating that HIF-1α was not involved in the increase in H3K9me2 by hypoxia challenge and by hypoxia mimetics.

**Hypoxia decreases the expression of Mlh1 and Dhfr genes and increases H3K9me2 in their promoters.** H3K9me2 is usually associated with gene repression and silencing (2). Previously, it has been reported that H3K9 methylation is connected with the repression or silencing of Dhfr (dihydrofolate reductase) and Mlh1 (involved in mismatch repair) genes (30, 31). It is of our interest to study whether the hypoxia-induced H3K9me2 is involved in the repression of these two genes. As shown in Fig. 6A, hypoxia decreased the mRNA levels of Mlh1 and Dhfr genes in a time-dependent manner. Next, ChIP assays were used to analyze their promoter regions following hypoxia challenge. Consistent with the repression of gene expression, the H3K9me2 levels at the promoter regions of Mlh1 and Dhfr genes were significantly increased following hypoxia challenge (Fig. 6B). We have reported previously
that hypoxia increased the expression of Cap43/Ndrg1 (a HIF-1-responsive gene) as early as 4 hours after challenge (24). It was found that the H3K9me2 levels at the promoter region of Cap43 gene remained unchanged (Fig. 6A). Collectively, these findings indicate that the increase in H3K9me2 is likely involved in the repression of genes during hypoxic stress.

Discussion

Hypoxia is commonly present in many solid tumors and often occurs when the growth of tumors outstrips their blood supply (32). In response to hypoxia, tumor cells increase the expression of genes related to angiogenesis, glycolysis, and glucose uptake using the transcription factor HIF (18). Hypoxia also decreases the expression of several genes, such as cellular adhesion proteins and DNA repair proteins, and leads to genetic instability and metastasis of tumor cells (33–36). In agreement with these reports, several clinical studies have shown that tumor oxygenation is an important marker for cancer patients’ prognosis and future distant disease recurrence (37, 38). Here, we report that hypoxia increases H3K9me2, an important repressive epigenetic mark. Our results suggest that this increase in H3K9me2 is likely linked with the repression of several genes during hypoxic stress.

H3K9-specific methyltransferase G9a is identified as one important cause of the hypoxia-induced H3K9me2. This conclusion is based on several lines of evidence, including that hypoxia increased both G9a protein and HKMT activity as well as genetic ablation of G9a significantly mitigated the hypoxia-induced H3K9me2. It was found that H3K9me2 and G9a protein levels were slightly increased in A549 control samples at later time intervals (Fig. 2B). These increases were likely caused by pericellular hypoxia as a result of increased cell densities in nontreated cells, which had already reached 80% to 90% confluence before hypoxia challenge. In support of this hypothesis, several studies reported that HIF-1α protein levels and the expression of HIF-regulated genes were increased in numerous mammalian cell lines when their cell density reached confluence (39, 40). We had also found that the expression of Cap43/Ndrg1, a HIF-regulated gene (24), was elevated in confluent A549 cells (data not shown).

Our results indicate that hypoxia increases G9a protein levels by post-translational mechanisms. In support of this notion, it was found that hypoxia did not increase G9a mRNA levels and an inhibition of general protein synthesis failed to block the increase of nuclear G9a protein in the hypoxia-exposed cells. In fact, hypoxia decreased G9a mRNA levels at later time interval, which was likely caused by the general inhibitory effects of lowered Ac-H3K9 and H3K9me2. It was reported that LSD1 demethylated H3K9me2 when lysine demethylation processes contributes to the hypoxia-induced H3K9me2. Hypoxia has also been shown to activate multiple signaling pathways in cells, such as mitogen-activated protein kinases and phosphatidylinositol 3-kinase (42, 43). However, several chemical inhibitors of these signaling pathways failed to block the hypoxia-induced H3K9me2 in A549 cells (data not shown). At present, the precise mechanism by which hypoxia increases nuclear G9a protein remains unknown.

In addition to the increases in G9a protein and methyltransferase activity, our results also indicate that the inhibition of histone lysine demethylases processes contributes to the hypoxia-induced H3K9me2. It was reported that LSD1 demethylated H3K9me2 when associated the androgen receptor in human LNCaP prostate tumor cells (12). However, this mechanism is not responsible for H3K9 demethylation in A549 cells because the androgen receptor gene is not expressed in A549 lung cells (data not shown). Moreover, H3K9me2 was not increased in A549 cells following treatment with deprenyl hydrochloride, a monoamine oxidase inhibitor that has been shown to abolish LSD1 activity in a previous study (12). A new class of H3K9 demethylases have just being identified recently. These H3K9 demethylases, JHDM2A and JMJD2A-D, all possess JmjC domains similar to the one found in the JHDM1 and require iron and 2-oxoglutarate to catalyze oxidative demethylations (15, 16). JHDM2A demethylates both H3K9me1 and H3K9me2 in vivo (16), whereas JMJD2A mainly demethylates H3K9me3.
Because oxygen is essential for the oxidative demethylation reactions catalyzed by these demethylases, oxygen deprivation should inhibit the activity of these H3K9 demethylase(s). In support of this notion, the hypoxic conditions used in this study inhibited HIF-related prolyl hydroxylases that belong to the same enzyme family as these H3K9 demethylases and thus resulted in the stabilization and accumulation of HIF-1α protein (Fig. 1A). Interestingly, hypoxia was found to increase both H3K9me2 and H3K9me3 in this study. At the time this article was written, the iron- and 2-oxoglutarate-dependent histone demethylases have been found to remove the methyl groups from H3K9 and H3K36 residues, but the list of their potential substrates is likely to grow. Hypoxia should also inhibit the demethylases for H3K36 or other unrecognized lysine sites. However, because the

**Figure 4.** Hypoxia increased global H3K9me2 levels by both G9a-dependent and G9a-independent mechanisms. **A**, WT, G9a<sup>−/−</sup>, and G9a<sup>−/−</sup> + G9α WT MES cells were exposed to hypoxia for 6 hours. The histones were extracted and immunoblotted for H3K9me2 and Ac-H3K9. **B**, HEK293 cells were replenished with either complete or methionine-deficient DMEM and incubated for 4 hours before hypoxia exposure. After hypoxia challenge for 24 hours, histones were extracted and immunoblotted for H3K9me2.

**Figure 5.** Hypoxia mimetics increased global H3K9me2 levels as well as G9a expression and activity. **A**, A549 cells were exposed to hypoxia, 100 μmol/L deferoxamine (DFX), or 1 mmol/L dimethylaspartylglycine (DMOG) for 18 hours. Western blottings for G9a and H3K9me2 were done as described earlier. **B**, HEK293 cells were transiently transfected with pEGFP-hG9a or pEGFP-hG9aΔSET expression vectors. Twelve hours after transfection, cells were exposed to 100 μmol/L deferoxamine for 18 hours. The methyltransferase activity of the overexpressed proteins was measured as described in Fig. 3. Columns, mean from the relative values of three independent experiments; bars, SD. *, P < 0.05, statistically significant change compared with control samples. **C**, A549 cells were seeded in F-12K complete medium. After overnight of incubation, the cells were replenished with either complete or methionine-deficient DMEM and incubated for another 4 hours. After exposure to 100 μmol/L deferoxamine for 24 hours, histones were extracted and immunoblotted for H3K9me2. **D**, HIF-1α<sup>+/+</sup> and HIF-1α<sup>−/−</sup> mouse embryonic fibroblast cells were exposed to hypoxia for 6 hours. Histones were extracted and immunoblotted for H3K9me2. In parallel samples, intracellular HIF-1α protein levels were measured with Western blotting.
demethylation processes for histone lysines are only one arm of their methylation metabolism, the global methylation levels are also affected by the effects of hypoxia on the other arm, the methylation processes. In other words, if the expression/activity of specific methyltransferases is decreased during hypoxic stress, the global levels of the corresponding histone modification may not change or only slightly increase, although the demethylases are inhibited. The possible effects of hypoxia on other histone lysine marks are not investigated here and should be examined in future studies.

Hypoxia was also found to increase H3K9me2 at the promoter regions of several genes. The increases in H3K9me2, together with the hypoxia-induced decrease in histone acetylation, may act in concert to repress gene expressions during hypoxic stress. In agreement with this notion, hypoxia was also found to decrease the histone acetylation at the promoter region of *Mlh1* gene in several mammalian cell lines (35), and genetic ablation of *G9a* alone did not attenuate the decrease of *Mlh1* mRNA levels during hypoxic stress (data not shown). As a repressive epigenetic mark, H3K9me2 is critical for the establishment of DNA methylation and long-term gene silencing (44). In a previous study on transgenes, Mutskov et al. (45) reported that both histone deacetylation and loss of H3K4 methylation were early events of the transgene silencing, whereas H3K9 and DNA methylation only appeared in the late phase of the silencing process. In contrast, a recent study on an endogenous gene silencing process reported that both H3 deacetylation and H3K9 methylation occurred within the same time window as gene inactivation, and these changes preceded the establishment of DNA methylation at the promoter region of this gene (46). Our results are consistent with the gene silencing process described in the second scenario because an increase of H3K9me2 occurred before or concurrent with the repression of genes during hypoxic stress. The persistence of hypoxia may eventually lead to the silencing of genes (such as *Mlh1*) that indirectly cause other gene mutation and genome instability and transform cells into a more aggressive phenotype.

In conclusion, this study provides the first evidence to our knowledge that hypoxia exposure increases global H3K9me2, which plays an important role in gene repression during hypoxia. This increase of global H3K9me2 by hypoxia is likely to be a key event in selection for the more aggressive phenotype of tumor cells.

Acknowledgments

Received 1/10/2006; revised 7/9/2006; accepted 7/12/2006.

Grant support: National Institute of Environmental Health Sciences grants ES00280, ES10344, and T32-ES07324 and National Cancer Institute grant CA16087.

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We thank Juliana Powell for her secretarial support and Martin Walsh for providing pEGFP-G9a and pEGFP-G9a(ΔSET) expression vectors.

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