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Nitric oxide is an epigenetic regulator of histone post-translational modifications in cancer

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Nitric oxide (nitrogen monoxide, NO) is an endogenously produced signaling molecule in cancer that regulates gene expression and cell phenotype. In recent years, new evidence has emerged regarding the roles of NO in epigenetic regulation and specifically of histone post-translational modifications (PTMs). Epigenetic effects of NO are mediated through transcriptional regulation of histone-modifying enzymes and by the ability of NO to modulate the activities and cellular localizations of these enzymes through the formation of iron–nitrosyl complexes and *S*-nitrosothiols.

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Epigenetics

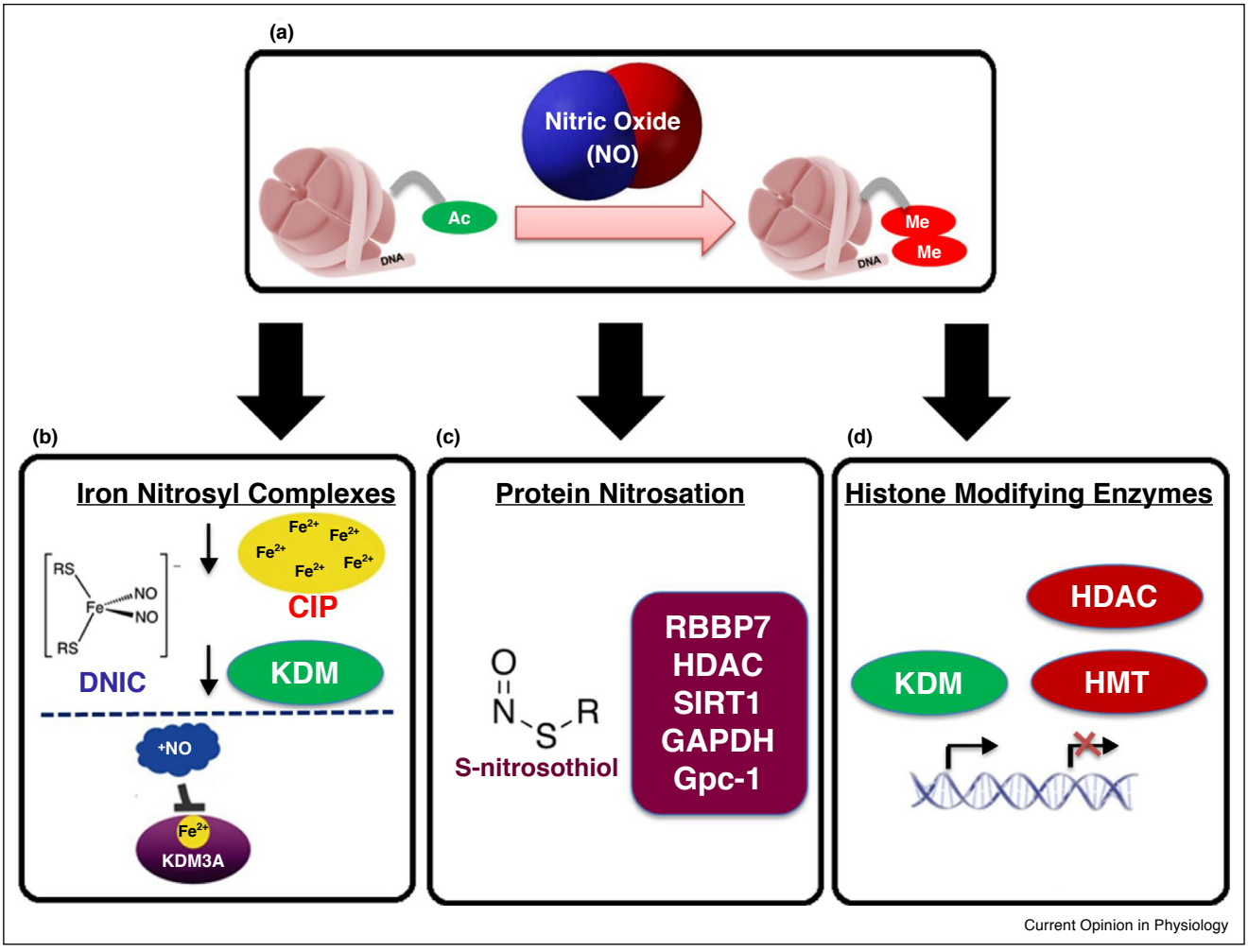
Epigenetics was initially defined as the process in which a fertilized zygote develops into a mature organism [1]. After over 50 years of rapidly expanding research into this field, the modern definition of epigenetics is the study of heritable changes in gene function that are not a result of variations in DNA sequence. Epigenetic changes must also involve a temporary mechanism different from the one required to maintain them from generation to generation. Mechanisms of epigenetic inheritance are predominantly mediated by DNA methylation, histone modifications, chromatin remodeling, histone variants, and non-coding RNA, among others [2]. In addition to being a vital component of developmental biology and normal physiology, epigenetic signatures are associated with differentiation, pluripotency, and cancerous states [3]. Understanding the origin of these signatures and their maintenance in inheritance could lead to significant changes in our understanding of many diseases and aid in the discovery of novel therapies.

Histones are alkaline proteins that bind negatively charged genomic DNA into compact structures called nucleosomes which allow DNA packaging and the formation of chromosomes. There are four types of core histones, H2A, H2B, H3, H4, and the linker histone H1 (Figure 2). The core histones exist as two dimers, H3–H4 and H2A–H2B, which are combined to form an octamer [4]. DNA wraps around this structural unit to form the nucleosome. However, in addition to providing a physical structure for DNA packaging, the C-terminal tails of these histones undergo extensive post-translational modifications regulated by histone-modifying enzymes [5]. This is important as specific modifications of amino acid residues on core histones can significantly impact chromatin structure and gene expression.

Nitric oxide

Joseph Priestly first characterized nitric oxide (NO, nitrogen monoxide) in 1772, but the molecule was not known to be synthesized in the human body until almost 200 years later [6]. NO is now known as an important free radical signaling molecule that regulates a variety of physiologic functions including vascular smooth muscle relaxation, host defense, and neurotransmission [7–9]. Our current understanding of NO, however, goes beyond these normal physiological processes to include its involvement in the etiology of various disease states such as obesity, endocrine disorders, inflammation, Alzheimer's disease, and cancer [10–13]. NO is uncharged, highly diffusible, and has a short biological half-life ranging from a few milliseconds to >2 s [14]. Although NO is a free radical, when compared to other biologically relevant free radicals, it is fairly unreactive. Under normal biological conditions NO only interacts with two types of molecules: other free radicals such as oxygen (O_2) and superoxide (O_2^-), or transition metals, principally ferrous iron (Fe(II)) [15]. In mammalian systems there are several physiological sources of nitric oxide. It is primarily produced by a pathway involving the enzyme nitric oxide synthase (NOS), which uses L-arginine and oxygen to catalyze the generation of NO and citrulline. The three isoforms of NOS are neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). The constitutively expressed nNOS and eNOS are activated by increases in intracellular calcium levels that stimulate calmodulin binding to the enzyme, resulting in the generation of low steady-state concentrations of NO. Conversely, the expression of iNOS is induced by cytokines and leads to considerably higher rates of NO production

Figure 1



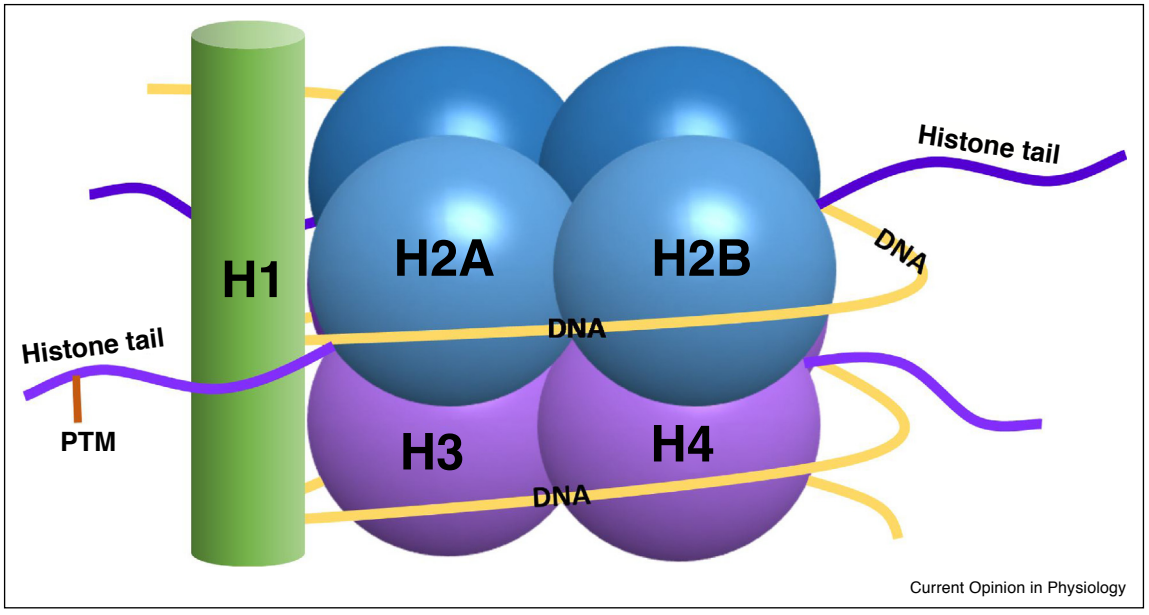
Epigenetic effects of NO on histone PTMs.
(a) Nitric oxide regulates the histone PTM landscape through various mechanisms both direct and indirect. **(b)** Metal interactions: inhibition of JmjC-domain histone demethylases (KDM) via formation of dinitrosyliron complexes (DNICs) that reduce the iron cofactor availability or through formation of an iron–nitrosyl complex in the active pocket of the enzyme. **(c)** *S*-Nitrosothiol formation on cysteine thiols of chromatin-modifying enzymes can inhibit their activities or alter their cellular localization. **(d)** Changes in the expression levels of histone-modifying enzymes reduce their global activities.

than nNOS or eNOS [16]. Signaling effects of NO are mediated by several mechanisms that involve either its direct reaction with the iron centers of proteins or via the formation of protein adducts that contain nitrogen oxide functional groups (i.e. *S*-nitrosothiols (RSNOs), 3-nitrotyrosine (3NT), or dinitrosyliron complexes (DNICs) [17,18]. Each of these mechanisms has been shown to directly or indirectly regulate epigenetic processes including histone PTMs (Figure 1).

Iron: iron–nitrosyl complexes

Some of the most important biological activities attributed to NO result from its covalent binding to the ferrous heme in proteins such as soluble guanylate cyclase (sGC) to form a five-coordinate heme-nitrosyl [19]. The effect of heme-nitrosyl formation can either increase or decrease the catalytic activities of specific proteins. Dinitrosyliron complexes, however, have the chemical formula $[Fe(NO)_2(SR)_2]$ and are formed intercellularly by the reaction of NO with thiols and iron, specifically the chelatable iron pool (CIP). Exposure of cancer cells to NO results in the quantitative conversion of the CIP into DNICs. DNICs are the most abundant NO-derived cellular adduct and have been shown to exhibit antioxidant properties as well as preserve the biological lifetime of NO [20–22]. Importantly, however, DNIC assembly sequesters iron from the CIP resulting in an iron-starved phenotype. Because the CIP is the dominant source of iron for many non-heme dioxygenases, DNIC formation therefore indirectly decreases the activities of numerous epigenetic enzymes by a reduction in this iron cofactor.

Figure 2



Histone octamer and linker histone.
The histone core is an octamer composed of two dimers, H2A–H2B and H3–H4. DNA wraps around this octamer and chromosomes are formed with the help of linker histone H1. Amino acid residues on the core histone tails undergo post-translational modification (PTM), ultimately resulting in differences in gene expression.

It was recently demonstrated that NO can regulate the types and locations of numerous histone PTMs such as methyl-lysine and acetyl-lysine residues [23•]. Addition or removal of these histone PTMs is catalyzed by a diverse set of enzymes. For example, the majority of histone demethylation is catalyzed by the JmjC-domain-containing class of histone demethylases (KDMs), which are non-heme iron dioxygenases. We demonstrated that in cancer cells, iron sequestration by DNIC formation reduced the activities of KDMs, which resulted in significant increases in global histone lysine methylation at numerous sites on core histones [24]. This effect could be reversed by supplementing cells with iron to augment their CIP. One lysine residue, the dimethyl form of histone 3 lysine 9 (H3K9me2, a gene silencing PTM), increased significantly in response to NO. This histone PTM also became enriched around the promoter regions of genes that were downregulated by NO, suggesting a causal link between change in histone PTMs and gene expression. Inhibition of KDMs has also been shown to play a role in immune cancer therapy. Specifically, H3K4me3 (a gene activating PTM) increased substantially and became enriched at promoters regions when KDM5A was inhibited. This resulted in open chromatin formation and the initiation of processes regulating innate memory in human monocytes [25].

Although DNIC assembly could *indirectly* inhibit KDM activity, it was also found that NO could *directly* inhibit the catalytic activities of these enzymes by binding to the non-heme iron in the catalytic pocket [24]. KDM inhibition required low nM concentrations of NO and was also reversible. Since methylation and acetylation at any given lysine residue are mutually exclusive, NO-mediated increases in methylation resulted in a concomitant decrease in global acetylation. This highlights how NO can significantly alter the global epigenetic histone landscape via multiple mechanisms. Not only did NO cause a reduction in acetylation but it resulted in redistribution of the acetyl marks throughout the genome. For example, NO enriched histone 3 lysine 9 acetylation (H3K9ac, a gene activation histone mark) around promoter regions of genes that were upregulated by NO [23•]. Together these mechanisms provide a link between NO, iron, changes in histone PTMs, and downstream gene expression changes.

Protein nitrosation: *S*-nitrosothiols

S-Nitrosothiols are protein or peptide cysteine thiols that contain a nitrogen oxide functional group. The dominant process for RSNO formation (nitrosation) under biological conditions remains controversial, but the result is the formal addition of a nitrosonium ion (NO^+) to a thiol. Since NO cannot directly react with a reduced thiol (RSH) to form an RSNO, it requires that either the NO or the thiol must first be oxidized [26]. Therefore, signaling effects of NO mediated by RSNOs are deemed *indirect* effects of NO since nitrogen oxides, other than NO itself, are ultimately involved in triggering the

responses. Like many post-translational modifications, RSNOs exert signaling effects by either activating or inactivating proteins or through their interactions with other PTMs such as phosphorylation and ubiquitination [27]. One group recently discovered that *S*-nitrosation of a histone binding protein, RBBP7, affected the structure and organization of the NurD complex, a group of proteins with chromatin remodeling and histone deacetylase activities [28•]. Others have shown that nitrosation of SIRT1, a deacetylase, inhibits its activity by causing the release of Zn^{2+} from a conserved Zn-tetrathiolate cluster [29•]. Nitrosation of cysteine residues at key sites on histone deacetylases (HDACs) has been shown to modulate their activity. For example, *S*-nitrosation of HDAC2 inhibited its deacetylase activity and consequently impaired its ability to bind DNA [30]. In addition to influencing HDAC activity, *S*-nitrosation of HDAC4 and HDAC5 facilitated their nuclear translocation [31]. In oral squamous cell carcinoma, hyperacetylation of histone H3 correlated with NO production. This was found to be mediated by *S*-nitrosated GAPDH, which increased its nuclear translocation and enhanced the activity of a histone acetyltransferase (HAT) protein by autoacetylation [32].

Gpc-1, a heparin sulfate proteoglycan ubiquitously expressed in vertebrate tissues, can be *S*-nitrosated in a copper-dependent reaction. These RSNO groups were shown to catalyze the cleavage of heparin sulfate chains, resulting in the release of an oligosaccharide containing reduced terminal anhydromannose (HS-anMan) [33]. Nucleolar glycosaminoglycans (GAGs), like HS-anMan, were shown to suppress N-terminal acetylation in histone H3 by inhibiting histone acetyltransferases (HATs) [34]. In support of this mechanism, treatment with XylNapOH, a primer for GAG synthesis, selectively lowered the level of H3 acetylation in T24 bladder carcinoma and HCC70 breast carcinoma cells without affecting the expression of histone H3 [35].

Indirect mechanism of NO: transcriptional regulation of histone-modifying enzymes

As we have discussed, histone-modifying enzymes covalently alter histone residues most notably by way of methylation, acetylation, phosphorylation, ubiquitylation, or sumoylation. These modifications recruit chromatin remodeling complexes and other histone modifiers which alter chromatin structure to either enhance or diminish gene expression [36]. As NO has been shown to change the expression levels of numerous histone-modifying enzymes, this constitutes another mechanism whereby NO can influence gene expression and cell phenotype epigenetically. For example, histone methylation status is regulated by several families of histone methyltransferases and demethylases which add or remove methyl marks from specific histone lysine residues. One study showed that all nine of the known H3K9 demethylases were upregulated at the mRNA level in response to NO. The upregulation of at least one demethylase, KDM3A, was found not to be a result of canonical NO signaling pathways: HIF-1α accumulation or soluble guanylyl cyclase activation [24]. NO also upregulated the mRNA levels of JmjC, a protein domain that is conserved among most of the KDMs [37].

Similarly, the expression levels of histone methyltransferases are differentially controlled by NO. Upon treating cells with NO, the expression levels of SETDB2 and SUV39H2 increased while the levels of G9a decreased [24]. This implies that the effects of NO on histone-modifying enzymes are context-specific and that even enzymes in the same class may differentially alter specific lysine residues-based solely on their expression levels.

NO has also been shown to epigenetically regulate histone deacetylases, which are enzymes responsible for removing acetyl groups from lysine tails typically resulting in a decrease in gene expression. One study analyzing the effects of cigarette smoke (which has high levels of NO [38]) on cytokine release in macrophages observed that protein expression of HDAC2 and HDAC3 and mRNA expression of HDAC5 and HDAC8 were decreased in bronchial biopsies and alveolar macrophages from COPD patients. At the same time, levels of smoke-induced release of IL-8 and NF-KB activation increased, suggesting the initiation of a proinflammatory state [39]. Because increases in inflammation in bronchial epithelial cells is associated with lung tumors [40], this could provide a mechanism whereby NO mediates cancer progression. In another study that focused on hypertension, cardiac remodeling, and fibrosis, it was found that treatment of H9C2 myoblasts with L-NAME (a non-selective inhibitor of nitric oxide synthase) upregulated the protein expression of both HDAC1 and HDAC2 [41•]. Another study looked at the role of NO in regulating HDAC expression in colonic inflammation. Researchers induced colonic inflammation in tissues by 2,4,6-trinitrobenzene sulfonic (TNBS) acid and observed an increase in expression levels of intracellular adhesion molecule-1 (ICAM-1) by way of increasing the nuclear translocation of transcription factor NF-KB. They found that the increased interactions between NF-KB and DNA were due to the transcriptional downregulation of global HDAC3. This resulted in an increase in acetylation of the H3K12 on the *Icam-1* promoter, opening the chromatin structure and facilitating access of NF-KB to its binding sites on the DNA. When inflamed tissues were treated with the transnitrosating agent GSNO, a global increase in HDAC3 expression was observed along with increased binding of HDAC3 to the *Icam-1* promoter, suppressing NF-KB binding to DNA. These results show that NO is able to change the expression levels of HDACs, resulting in anti-inflammatory effects during colonic inflammation [42]. The differential effects of NO on inflammatory

responses mediated by HDACs highlights that NO can exhibit contradictory responses that may depend on microenvironmental factors which exist across various disease states.

While NO affects the transcription of histone-modifying enzymes, these enzymes can also regulate the transcription of genes coding for nitric oxide synthase enzymes. For example, a recent study in tumor-induced MDSCs (myeloid-derived suppressor cells) showed that iNOS expression was activated by an increase in the expression of the histone methyltransferase SETD1B. This enzyme can trimethylate H3K4, which the researchers showed was enriched at the *nos2* promoter region, resulting in a subsequent increase in iNOS expression. Targeting SETD1B expression in MDSCs could therefore be a therapeutic approach to inhibit immune suppression and improve the efficacy of cancer immunotherapy [43•]. These results underscore not only that NO can regulate histone PTMs, but that histone PTMs can potentially mediate NO production by regulating the expression of iNOS.

Conclusions

The epigenetic effects of NO in cancer are partially mediated by histone PTMs through both direct and indirect mechanisms. These mechanisms include reactions of NO with the various forms of cellular iron (iron–nitrosyl, DNIC) as well as the formation of protein adducts containing nitrogen oxide functional groups (*S*-nitrosothiols). In addition to altering histone PTMs by directly affecting the activities of chromatin-modifying enzymes, NO can also mediate the expression levels of these enzymes. This constitutes an important but indirect mechanism for NO to influence steady-state histone PTM levels. As histone PTMs are a dominant upstream driving force regulating gene expression, the effects of NO on histone PTMs have the potential to alter the global epigenetic landscape resulting in significant changes in gene expression and cell phenotype. Therefore, in addition to the canonical and well-documented roles of NO in cancer etiology and cell signaling, epigenetic regulation of histone PTMs should be considered when trying to evaluate the totality of potential signaling responses attributed to NO in the context of cancer pathobiology and treatment.

Conflict of interest statement

Nothing declared.

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