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The role of nitric oxide in melanoma

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Abstract

Nitric oxide (NO) is a small gaseous signaling molecule that mediates its effects in melanoma through free radical formation and enzymatic processes. Investigations have demonstrated multiple roles for NO in melanoma pathology via immune surveillance, apoptosis, angiogenesis, melanogenesis, and on the melanoma cell itself. In general, elevated levels of NO prognosticate a poor outcome for melanoma patients. However, there are processes where the relative concentration of NO in different environments may also serve to limit melanoma proliferation. This review serves to outline the roles of NO in melanoma development and proliferation. As demonstrated by multiple in vivo murine models and observations from human tissue, NO may promote melanoma formation and proliferation through its interaction via inhibitory immune cells, inhibition of apoptosis, stimulation of pro-tumorigenic cytokines, activation of tumor associated macrophages, alteration of angiogenic processes, and stimulation of melanoma formation itself.

Keywords

nitric oxide; melanoma

Mechanisms of nitric oxide production in melanoma

Nitric oxide (NO) is a free radical inorganic signaling molecule that is generated via multiple mechanisms (Figure 1). One of these mechanisms catalyzed by nitric oxide synthase (NOS) is the oxidation reaction of L-arginine and oxygen with a NADPH co-substrate to produce L-citrulline and NO [1]. There are 3 isotypes of NOS in humans: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). iNOS is transcriptionally induced by immunologic stimuli produced by various cells in the immune system (e.g. macrophages) in a calcium independent manner [2, 3]. The eNOS and nNOS isoforms have been collectively grouped as constitutively active NOS (cNOS). These cNOS isoforms are constitutively expressed and their activity has classically been thought to be dependent on prior calcium influx that enables the isoforms to bind to calmodulin. Normal melanocytes express both cNOS and iNOS [4].

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Another source of NO is the conversion of nitrate into NO in a two-step process. First nitrate is reduced to nitrite and then nitrite is reduced to nitric oxide. For example, oral bacteria reduce dietary inorganic nitrate into nitrite and then in an acidic environment such as the stomach nitrite is then reduced to NO via non-enzymatic disproportionation [5–7]. Therefore, it is not surprising that the major sources of nitrate and nitrite are the diet, products of the endogenous NOS pathway, and products of the oxidation and reduction by commensal organisms [1, 7]. A similar mechanism occurs in the skin where nitrate present in the sweat is reduced to nitrite with subsequent conversion to NO in the presence of the acidic skin surface [8]. Nitrite may also be converted into NO enzymatically via the xanthine oxidoreductase or proteins containing the iron porphyrin moiety (e.g. cytochrome c, deoxyhemoglobin, mitochondrial electron transport chain) [2, 9]. In normal human skin, photolabile compounds (e.g. nitrate, nitrite and the S-nitroso compounds such as S-nitrosothiols, S-nitrosoalbumin, S-nitrosoglutathione or S-nitrosocysteine) generated NO via photodecomposition after exposure to ultraviolet A (UVA) or blue light. In the presence of blue light, photolabile NO derivatives photodecompose to NO via a copper (Cu^{1+}) dependent mechanism. In the presence of UVA radiation, nitrite decomposed to NO and subsequent electron transfer reactions generated highly toxic nitrogen dioxide (NO_2^*), which in turn initiated a lipid peroxidation chain reaction [10, 11]. It is not surprising with these interdependent processes nitrite can also serve as the substrate to form s-nitrosothiols in vivo [12]. NO and metabolites of NO such as nitrite may be measured via colorimetric dyes such as the Griess reagent, fluorescent dyes such as DAF-FM, and in vivo via electron paramagnetic resonance spectroscopy [13–15]. Generally, NO may be derived enzymatically (NOS, xanthine oxidoreductase, or iron porphyrin containing proteins) or non-enzymatically (UVA or blue light photochemistry or low pH induced disproportionation).

Nitric Oxide Biochemistry

NO exerts its effects through two major signaling pathways, the cGMP dependent pathway and the cGMP independent pathway (Figure 2) [16]. In the cGMP dependent pathway, NO synthesized from NOS diffuses across the plasma membrane and cytoplasm, reacts with the active site of soluble guanylate cyclase and produces cGMP. cGMP then phosphorylates the cGMP dependent protein kinase G, which in-turn phosphorylates and activates cGMP dependent gated ion channels and phosphodiesterase. As describes above, NO and its derivatives may be involved in redox chemistry with other small molecules and metal complexes on proteins [6, 17]. The cGMP independent pathway occurs commonly through modification of proteins through S-nitrosylation of cysteine residues [16, 18]. Nitrosation or nitrosylation (biological term for nitrosation) is the incorporation of NO onto another molecule with an alkene bond between the N and O. Such post translational modifications affect transcriptional activity by alteration of DNA-protein interactions [16, 19].

The derivatives of NO are responsible for multiple post-translational modifications resulting in altered functional properties of proteins [2]. NO can react with superoxide anions to yield peroxynitrite, which in turn serves as a nitrating and nitrosylating agent. Peroxynitrite interacts with amino acids, generating nitrotyrosine and reversible thiol nitrosylation [20]. In fact, there are known motifs in proteins that have been shown to be preferentially nitrosylated [18]. A literature review of 233 S-nitrosylated proteins revealed that S-

nitrosylation sites were found in alpha helices, solvent accessible areas, and structural motifs with charged amino acids within 6 angstroms [21]. S-nitrosylated proteins are also involved in the reactions of energy metabolism such as mitochondrial respiration/proliferation and reactions of glucose metabolism [21, 22]. To review, researchers have divided S-nitrosylation mechanisms into NOS dependent and independent pathways [23]. NO itself can diffuse intracellularly and bind to a heme moiety of guanylate cyclase. This event leads to enzymatic activation and subsequent formation of cyclic GMP and signaling via the MAPK cascade and JAK/STAT pathway [24]. Nitration is the incorporation of the NO₂ moiety via peroxynitrite intermediates most often on tyrosine residues. It is likely that peroxynitrite binds to carbon dioxide and this nitrosoperoxy carbonate radical then reacts with tyrosine residues to form 3-nitrotyrosine [17]. Like protein nitrosylation, common motifs for nitration have been identified and there is a computational prediction program known as GPS-YNO2 to predict tyrosine nitration sites on proteins [25]. Other protein residues such as phenylalanine have been nitrated in vitro [26]. According to the described literature, protein nitration is considered to be an irreversible event, and nitrosylation is considered to be reversible. In general NO can be thought of as acting on biological pathways via small molecule and protein interactions [6, 17, 19, 27].

Nitric oxide biochemistry was initially thought to be non-enzymatic in character. However, nitric oxide derivatives may bind to iron complexes to facilitate nitration at a later time. NO can be transported by hemoglobin to sites of low oxygen concentrations (e.g. rapidly growing tumors), and then can release NO to competitively inhibit proteins such as cytochrome c that also contain iron complexes [28]. Cytochrome c can both transport NO and itself be nitrated to mediate apoptosis of T cells [29]. Other metalloproteins besides cytochrome c containing ferric iron, iron sulphur clusters, copper or zinc ion structural motifs may also be the targets for NO [22]. NO biological effects can therefore be divided into whether they are dependent on transient elevations in nitric oxide concentrations (eNOS and nNOS) dependent on calcium fluxes within the cell, or more sustained elevations in nitric oxide levels (iNOS) [24]. From the above description of NO biochemistry, it is clear that NO may play roles in the immune system, cell death, and cell proliferation. This paper describes the roles of NO within the context of melanoma.

Sources of nitric oxide in melanoma

Historically, iNOS was found to be overexpressed by macrophages, but NO produced by iNOS was found to be important in the proliferation in melanoma cell lines such as A375, WM94, YU SAC2, and YUZA6 [30, 31]. iNOS expression is higher in melanoma cells than normal melanocytes and the expression level increased with increased Clark stage of melanoma [32, 33]. This is significant as increasing Clark stage represents an increased thickness of the melanoma and is associated with an increased risk of metastasis [34]. Interestingly, the expression of iNOS in melanoma tumor specimens can range from completely negative to nearly 100% positive in all cells [30]. This finding of increased iNOS expression is not limited to cutaneous melanoma. In other subsets of melanoma such as choroidal melanoma there is increased expression of iNOS activity in melanoma cells compared to melanocytic nevi [35]. Cutaneous melanoma lesions expressed iNOS within the papillary dermis and in the walls of blood vessels [32]. While, in metastatic lesions, iNOS

expression was more intensely stained in neoplastic cells at the periphery of the tumor mass than the center [32, 33]. Whereas, in lymph nodes with confirmed metastasis, the NO staining was greater in the marginal sinus than the lymphoid parenchyma [32]. From this study we can conclude that the expression of iNOS is involved in the early steps of the conversion from a melanoma contained within the skin to a metastatic lesion [32]. In cutaneous melanoma samples from patients, iNOS expression was also found to be high in basal and suprabasal epidermal layers, adnexal epithelium, and in macrophages. In some cases endothelial cells expressed iNOS, notably in areas of regression of melanoma and within the cytosol of presumed melanoma cells within the tissue sections [33]. Interestingly, there is an association between density of iNOS staining and higher lymphatic vessel density in melanoma samples [33]. The aforementioned investigations described the role of iNOS expression in melanoma, but nNOS protein has also been studied in melanoma cells. In one such study, 49% of benign nevi, 72% of atypical nevi, and 82% of primary malignant melanoma expressed nNOS [36]. Furthermore, an inhibitor of nNOS utilized in melanoma cell lines resulted in decreased intracellular levels of NO and reduced proliferation of melanoma cells [37]. It has been observed that NO can be produced by the melanoma tumor cells by themselves or by cells in the tumor environment. In BRAF mutant A375 melanoma cell lines have low production of NO production at baseline. In this case, NO diffuses from macrophages and other immune cells to exert its effects on the tumor cells [38]. Interestingly, in the tumor microenvironment, high levels of NO exert cytotoxic effects whereas low levels of NO promote tumor growth [16]. Therefore, the notion has been introduced that nitric oxide has both pro-tumorigenic and anti-tumorigenic roles [39]. A discussion of the role of NO in the immune system, apoptosis (and general cellular cytotoxicity) of melanoma cells, initiation of melanoma growth, angiogenesis, development of metastases, and thoughts for potential therapeutic interventions in melanoma is the subject of this review of nitric oxide in melanoma.

Production of NO by tumor associated macrophages is increased by melanoma cells

NO influences the inflammatory microenvironment of melanoma to facilitate tumor cells escape from the immune system. For example, human ulcerated melanomas that are prone to worse patient outcomes demonstrated significantly higher percentage of intratumoral iNOS-positive tumor associated macrophages (TAMs) in comparison to melanomas that were found to be nonulcerated [40]. Murine melanoma tumor cells directly stimulated macrophages to release NO by cell to cell interaction via IFN- γ dependent pathways [40, 41]. Although first observed in a lung cancer murine model, another possible mechanism by which NO mediated tumor growth is inhibition of the IL-12 tumor regressing cytokine by macrophages and subsequent growth of melanoma in murine models [42, 43]. DC-derived macrophages may also utilize NO to inhibit CD8⁺ T cell proliferation and facilitate immune escape [44]. Synthetically, the triterpenoid, CDDO-Imidazolidine inhibits NO production by iNOS in murine macrophages and inhibits B16 murine melanoma growth [45]. Interestingly, NO can also mediate melanoma tumor regression in certain circumstances. Immune cells such as NK cells may facilitate production of high levels of production of NO by macrophages to kill B16 melanoma cells [46]. In addition cytotoxic T cells are recruited to the melanoma cells (MeWo) after low dose radiation by iNOS⁺ macrophages [47]. There are

clearly scenarios where macrophages are responsible for increased or decreased NO concentration through the actions of macrophages.

Myeloid Derived Suppressor Cells (MDSCs) promote immune suppression via NO dependent mechanisms

MDSCs are a class of immature myeloid cells that are typically subdivided into monocytic (M-MDSC) and granulocytic (i.e. polymorphonuclear MDSC; PMN-MDSC) subsets that have immune suppressive functions on other immune cells to help cancers evade the immune response [48–50]. MDSCs were first found to have suppressive functions on T cells (CD8⁺ T cells) to blunt the immune responses via induction of iNOS and ARG1 by production of reactive nitrogen species [48]. In early experiments both wild type and iNOS KO mice had increased numbers of CD11b⁺Gr-1⁺ cells (now known as MDSCs) after treatment with granulocyte macrophage colony-stimulating factor (GM-CSF). However, immunosuppression as measured by inhibition of T cell proliferation by CD11b⁺Gr-1⁺ cells was observed only in the iNOS expressing mice [51]. Interestingly, in NOS2 KO mice with implanted B16 melanomas, MDSCs are unable to suppress T cell activity [51]. Similarly, a PDE-5 inhibitor (sildenafil) also inhibited decreased arginase and NOS2 expression as well as abrogated MDSC suppressive activity in B16 melanomas [52].

In melanoma, M-MDSC are elevated in the peripheral blood [49]. For example, in the B16 melanoma murine model, M-MDSCs produce NO and inhibit the proliferation of adoptively transferred CD8⁺ T cells and this inhibition of T cell proliferation was abrogated in the presence of an iNOS inhibitor (L-NMMA) [53]. Furthermore, NO produced by iNOS in the setting of low arginine (e.g. high Arginase expression) may produce reactive nitrogen species (e.g. peroxynitrite) and result in apoptosis of T cells [54]. Expression of the DC-HIL on M-MDSCs in the B16 model inhibits effector T cells by binding to syndecan-4 by a NO dependent mechanism [55]. Although, most models suggest that the monocytic population of MDSCs is important for melanoma there is at least one murine model driven by increased expression of the metabotropic glutamate receptor 1 (Grm1) that suggests that iNOS expression in granulocytic MDSC and presumed production of NO is responsible for decreased T cell responses [56].

MDSCs inhibit T-cell function in melanoma both at the tumor itself and peripheral lymphoid organs [49]. In the RET (REarranged during Transfection) transgenic murine melanoma model, NOS2 expression was associated with increased MDSC recruitment by IL-12 producing $\gamma\delta$ T cells and activation of T regulatory cells [57, 58]. VEGF secretion in the tumor microenvironment via iNOS dependent mechanisms also led to the recruitment of MDSC [59]. MDSC migration may also be inhibited by compounds such as OSU-53 (a thiazolidinedione) which reduces expression of iNOS within MDSCs derived from melanoma patients [60]. The calcium binding protein S100A9 also supported MDSC proliferation, recruitment to tumor and inhibited immune responses in cancer [61]. Tasquinimod abrogated S100A9 activity and inhibited MDSC suppression of T cells in a B16 melanoma model. Interestingly in this case, tasquinimod treatment increased M1 macrophage activity and iNOS expression [62]. Another recent mechanism of inhibition of MDSCs is that ibrutinib treatment in human MDSCs led to a reduction in NO production

and inhibition of MDSC generation from normal peripheral mononuclear blood cells. Ibrutinib also resulted in decreased numbers of total MDSC in a murine B16 melanoma model [63]. Melanoma may also utilize MDSCs to evade the immune system in the context of chemotherapy administration. Interestingly, low dose chemotherapy such as cyclophosphamide increased NO production via increased numbers of MDSC [64]. Multiple mechanisms have been proposed across different cancer types, but the specific mechanisms of NO control of MDSC activity in melanoma are not entirely clear.

NO modulates the production of cytokines and chemokines

Cytokines and chemokines, through nitric oxide mediated mechanisms promote chemotaxis of immune-inhibitory cells, angiogenic processes, and alter cellular processes such as apoptosis. It has been demonstrated that increased NO concentration in the tumor microenvironment is associated with downregulation of the CXCL10 chemokine which serves to limit recruitment of immune cells such as plasmacytoid dendritic cells. CXCL10 had increased mRNA expression levels in melanoma samples obtained from patients without detectable iNOS expression [65]. CXCL10 is also responsible for the recruitment of CD8⁺ T cells, natural killer cells, monocytes and macrophages to the tumor microenvironment [66]. Clinically, it is not surprising that interferon- α (IFN- α) therapy upregulates CXCL10 in the serum of melanoma patients [67]. Furthermore, increased nNOS mRNA expression in 12 melanoma cell lines correlated with suppression of IFN- α activity as measured by downstream phosphorylation of STAT1 in T cells (CD4⁺ and CD8⁺) and monocytes [68]. As expected, NONOate, a chemical that slowly releases NO and mimics NOS1 activity, induced a dose-dependent suppression of IFN- α p-STAT1 levels in T cells and monocytes that were cultured with the melanoma cell lines [68]. The suppression of IFN- α p-STAT1 activity by NO was reversed by specific NOS1 and NOS2 inhibitors in a dosedependent fashion [68]. Other cytokines such as IL-15 may actually slow melanoma tumor growth by increasing NO and IL-12 levels in macrophages contributing to apoptosis of individual melanoma cells [69]. Furthermore iNOS expressed in CD4⁺ T cells in the MT-RET1 melanoma model inhibits differentiation into T_{reg} cells by limiting the activity of the cytokine TGF β 1 [70]. However, when B16 melanomas designed for C57Bl/6 mice were injected in Balb/c mice with mesenchymal cells treated with the inhibitory cytokines IFN- γ and TNF- α , the melanomas are able to escape the immune system and cell death presumably by promoting iNOS expression in mesenchymal cells [71]. Decreased NO can therefore also be responsible for downregulation of cytokines. For example decreased NO concentration is associated with downregulation of angiogenic cytokines such as IL-8 in A375 and SB2 melanoma cell lines [65]. The angiogenic IL-8 cytokine has been shown to recruit suppressive MDSCs and have been reviewed elsewhere [72]. Other immune inhibitory cytokines such as IL-6 through NK/p38 MAPK pathway signaling, promoted AP-1 activity, and subsequently increased iNOS expression in B16 murine melanoma cells [73]. Therefore, the context in which NO is produced is important for modulating the levels of cytokines/chemokines and for determining melanoma cell survival or death.

There is evidence that nitric oxide both promotes and inhibits apoptosis

Apoptosis of eukaryotic cells was initially thought to be mediated via two major pathways, the intrinsic/mitochondrial pathway and the extrinsic/death ligand pathway. However, in the

final step of the cascade, the cell death is known to occur via a common pathway that includes activation of caspase 3. A third perforin or granzyme-dependent pathway utilized by cytotoxic T cells also leads to apoptotic cell death via caspase 3 activation [74]. NO plays a role in apoptosis of melanoma tumor cells and immune cells. Lymphocytes, in general, undergo apoptosis by nitration of key tyrosine residues inhibiting phosphorylation events [75]. In hypoxic murine melanoma models, IFN- γ activated macrophages synthesized NO in a HIF-1 (hypoxia inducible factor 1) and iNOS dependent fashion to promote apoptosis of melanoma cells [76]. Early reviews discussed the general roles of NO in apoptosis including up-regulation of the tumor suppressor protein p53, activation of caspases, and downregulation of BCL2. [77]. Similar processes are felt to be in play in melanoma models. Human A375-S2 melanoma cells treated with evodiamine (inducer of iNOS), increased NO production, phosphorylated p53 and promoted apoptosis likely via a p38 and NF κ B dependent mechanism [78]. The addition of a NOS inhibitor (L-NAME) reduced the numbers of cells in the subG1 and G2/M cell cycle phases and reduced the ability of p53 to promote apoptosis [78]. The oncogenic protein MED-27 possibly abrogates apoptosis through increasing iNOS expression [79]. Inhibition of NO led to cell apoptosis in patient derived melanoma cell lines. When these cells were treated with a NO inhibitor (aminoguanidine), the mitochondrial membrane potential was altered to favor apoptosis, expression of caspases (1, 3, and 6) increased, and BCL-2 expression levels decreased [80]. Interestingly, iNOS inhibition did not induce apoptosis in normal melanocytes as they did in melanoma cells derived from patients [80]. NO can also inhibit apoptosis by various pathways such as S-nitrosylation of activated caspases, kinases which are involved in activation of p53, possibly preventing assembly of the apoptosome [81]. Other NOS isoforms such as the nNOS isoform may also be responsible for melanoma cells escaping apoptosis. When nNOS expression was specifically reduced in melanoma cells, JunD, MMP-1, APE/Ref-1, and Bcl-2 expression increased leading to melanoma cell death presumably via apoptotic pathways [82]. Interestingly, different types of melanoma may have increased resistance to apoptosis as a direct function of NO activity. For example, amelanotic melanoma grown in hamster models demonstrated increased NO content and were more resistant to apoptosis compared to their melanocytic counterparts [83]. Additionally, mesenchymal stem cells lacking p53 (that are exposed to B16-F0 melanoma cells) in mice leads to upregulation of iNOS, increased immunosuppression, and in general increased tumor growth of melanoma cells [84].

Multiple natural products have been demonstrated to either promote or inhibit apoptosis in melanoma models. For example, capsaicin and resveretrol induced nNOS and iNOS production in A375 human melanoma cells and induced apoptosis via a cytochrome c dependent pathway [85]. In this case, the mechanism of apoptosis was facilitated via the extrinsic pathway including the death receptors DR4 and Fas (CD95) [85]. α -tocopheryl succinate, a derivative of vitamin E, also led to increase NO production and apoptosis in B16-F1 murine melanoma [86]. Physalin A induces apoptosis in A375-S2 melanoma cells via the common pathway of apoptosis (caspase 3) via iNOS dependent pathways. Interestingly, induction of autophagy suppressed NO production in melanoma cells and inhibited those cells from undergoing apoptosis [87]. Rice bran polysaccharides mediate apoptosis of B16 melanoma cell lines in a dose dependent fashion by stimulating the

production of NO likely in macrophages [88]. Similarly, B16-F1 melanoma cells in the pulmonary vasculature resulted in an increase in eNOS from pulmonary endothelial cells. NO concentration subsequently increased and resulted in apoptosis in melanoma cells [89]. In liver tissue, NO production in the sinusoids (endothelial cells and hepatocytes) is the source of NO for apoptosis of melanoma metastases [90]. Naturally occurring polyphenols (ceramide) decrease iNOS production by B16-F10 cells but increase eNOS production by the vascular endothelium. Subsequently there is increased NO and ceramide resulting in apoptosis of these cells. Interestingly, an extremely low or high concentration of NO decreases CCL2 expression, whereas intermediate levels of NO may increase BCL2 expression to inhibit apoptosis [91]. A naturally occurring naphthoquinone, 2-methoxy-6-acetyl-7-methyljuglone (MAM) induces apoptosis by activation of the JNK pathway with subsequent inhibition of iNOS expression and caspase dependent apoptosis [92]. Contrary to the effects of the natural product capsaicin, the natural product curcumin inhibited iNOS production of NO and resulted in apoptosis of melanoma cells [93]. Resveratrol abrogated the nitric oxide dependent apurinic/apyrimidinic endonuclease-1/redox factor-1 pathway that serves to promote expression of pro-growth genes such as Bcl-2 and MMP-1 [94]. Therefore, natural products have demonstrated to us that the relative concentrations of NO and the context in which it functions determines whether melanoma cells will undergo apoptosis.

Chemotherapy and targeted anti-cancer agents have also helped to elucidate the role of NO in apoptotic processes. In A375 melanoma cells, NO depletion followed by cisplatin treatment led to increased p53 and p21 levels with subsequent apoptosis via a cGMP independent process [95]. When iNOS is inhibited by L-NIL in A375 human melanoma cells lines, the cells become sensitive to VP-16 chemotherapy and undergo apoptosis as demonstrated by increased caspase 3 activation [96]. Similarly, when cisplatin is given to A375 cells after L-NIL administration BCL2 is downregulated and apoptosis is increased (TUNEL assay) [97]. Intercalating agents derived from anthracyclines such as WP-760, demonstrated inhibition of iNOS and likely activated the intrinsic mitochondrial apoptotic pathway [98]. Immune cells with increased NO levels may behave differently in the setting of chemotherapy. Dendritic cells (DCs) treated with a nitric oxide donor ex vivo and adoptively transferred into the B16-F1 model demonstrated resistance to apoptosis in the setting of cisplatin chemotherapy administration and administration of these cells resulted in slower tumor growth. The NO in the adoptively transferred DCs protected these cells from apoptosis by inhibiting the formation of the pro-apoptotic sphingolipid ceramid [99]. Targeted therapies such as vemurafenib (BRAF inhibitor) inhibited the growth of melanoma cells in part by inhibiting iNOS-dependent mTOR pathway activation [100]. β -blockers such as propranolol may decrease stress related increases in eNOS expression and subsequent B16 murine melanoma tumor growth [101]. Specific blockade of β 3-adrenergic receptors increased apoptosis in the setting of decreased iNOS activity [102]. In this section it was demonstrated that NO can both inhibit and activate apoptosis dependent on a multitude of factors via its three isoforms, eNOS, iNOS, and nNOS.

In some studies, the authors do not delineate apoptotic mechanisms of cell death or tumor growth but outline a clear association of NO with melanoma cell death

For example, B16-F10 melanoma cells undergo lysis in the presence of macrophages via a TLR7/8 and NO dependent process [103]. Increased NO concentrations in hepatic sinusoidal endothelium led to increased levels of γ -glutamyl-cysteinyl-glycine (GSH) and subsequently increased TNF- α mediated cell death of B16-F10 melanoma cells [104]. The activity of cytotoxic T lymphocytes was also enhanced via NO by inhibiting the HIF1 α pathway and subsequently decreasing inhibitory PD-L1 expression on B16-OVA murine melanoma cells [105]. Natural substances such as oligoesculin increased NO production in macrophages and promoted lysosomal activity with a subsequent reduction in tumor growth in B16-F10 melanoma models [106]. In contrast to the role that NO plays in promoting cytotoxicity, NO generated from MDSCs decreased the activity of cytotoxic T cells on melanoma cells. Addition of a NO scavenger (cPTIO) in this case led to a decrease in tumor growth [107]. Chemical compounds such as indole derivatives or methanol extracts of *Solanum muricatum* also inhibit NO production and human melanoma cell line growth [108, 109].

Environmental factors in the vicinity of melanoma cells can either inhibit or promote metastasis via alteration of NO metabolism

For example, hypoxic B16-F10 melanoma cells demonstrate increased number of lung metastases in murine models via suppression of the cGMP-dependent NO signaling pathway [110]. Some metastatic melanoma cell lines (e.g. K-1735) have increased iNOS mRNA expression compared to those that tend to metastasize to the lung [111, 112]. Interestingly, IFN- γ and LPS resulted in increased iNOS expression only in non-metastatic melanoma cell lines and transfection of iNOS into the metastatic cell lines resulted in fewer lung metastases [112, 113]. Inhibition of eNOS restored cell adhesion between melanoma cells and VCAM-1 allowing the melanoma cells to survive and after UVB radiation exposure [114]. Analogous to the murine experiments, human melanocytes stimulated with tumor necrosis factor- α , interferon- γ , or lipopolysaccharide increased iNOS expression but induction appears to be reduced in melanoma cell lines such as SK-Mel-19 or O-Mel-2 [115]. Contrary to the mouse cell lines, human melanoma cells lines express iNOS at relatively low levels [115]. Just as we have seen with other processes, NO may also promote changes in the microenvironment to support tumor growth. Metastatic melanoma murine cell lines such as B16-BL6 have increased iNOS expression and numbers of metastases as compared to its B16 parent cell line by inhibiting lymphocyte mediated tumor immunity [116]. Melanocytes decrease adhesion to extracellular matrix elements such as fibronectin in the presence of nitric oxide in a concentration dependent manner [117]. Furthermore, when normal melanocytes are immortalized using traditional transfection techniques with HPV16 viral genes E6 and E7, these cells also detach from the extracellular matrix in the presence of NO likely via a cGMP dependent process [118]. Increased levels of NO under inflammatory conditions also increased levels of matrix metalloproteinase I via MAPK-dependent pathways in C32TG and Mewo melanoma cell lines to alter the extracellular matrix thereby promoting melanoma metastases [119]. Interestingly, the expression of leptin (normally found in states of obesity) in the melanoma microenvironment may increase NO concentration and endothelial progenitor cell proliferation leading to increased angiogenesis and melanoma tumor growth [120].

Angiogenetic processes may be promoted or inhibited by NO

There is evidence that NO can increase angiogenesis and thus promote tumor progression. Most of this evidence is derived from the association between NO and VEGF expression. For example a photoactive inhibitor of NOS (NS1) decreased VEGF production in A375 melanoma and resulted in G2/M cell cycle arrest. In a complementary ex vivo model of angiogenesis, tube formation of HUVEC cells (sign of angiogenesis) was decreased in the setting of the NS1 compound [121]. Furthermore, it was shown in 50 choroidal melanoma samples from humans that there was an association between increased protein levels of iNOS, HIF-1 α , COX-2, and VEGF [35]. Using iNOS knock-out murine models, decreased iNOS expression inhibited the growth and metastases of B16 melanoma cells likely via a decrease of VEGF expression in the tumors and reduction in the tumor vasculature at the tumor invasive margin [122, 123]. VEGF inhibitors such as axatinib decreased both tumor growth and iNOS expression in B16-F1 murine melanoma models [124]. Naturally occurring isothiocyanates may be utilized to inhibit NO and TNF α production and inhibit tumor specific angiogenesis [125]. An interesting side note is that (+) catechin also inhibits angiogenesis by decreasing production of VEGF and inhibiting NO production in LPS-treated macrophages to potentially limit apoptotic processes [126]. In the A375 BRAF mutated human melanoma cell lines, VEGF directly increased iNOS protein levels and cell growth without affecting the protein levels of the nNOS and eNOS isoforms [127]. The eNOS and nNOS isoforms may have different effects on angiogenesis. Angiogenesis is promoted through adrenomedullin mediated eNOS signaling in melanoma [128]. In other studies it was suggested that B16-F10 melanoma cells utilize eNOS upregulation to promote VEGF mediated lymphangiogenesis [129]. On the other hand, inhibition of eNOS within the endothelial cells via a NOTCH dependent pathway may be the culprit for dysfunctional vascular formation and growth within tumors [130].

iNOS expression and increased NO production correlates with a poor prognosis in melanoma

As early as 2000, it was demonstrated that increased iNOS and nitrotyrosine levels were associated with poor survival in metastatic melanoma [131]. Immunohistochemistry staining of iNOS expression in tumors from 132 stage III melanoma patients demonstrated a strong association between iNOS expression and shortened survival time [132]. Similar studies performed by the Grimm laboratory demonstrated that iNOS expression was associated with a poor survival outcome [133]. Interestingly, subsets of melanoma that have increased proliferation rates such as BRAF mutated melanoma have also illustrated increased iNOS expression [134]. In patients with melanoma metastases to regional lymph nodes (stage III); there were decreased metastases to lung, liver and brain within three years in cases where the melanoma cells had decreased iNOS expression [135]. In another clinical trial of melanoma patients undergoing therapy with ipilimumab, increased NO production by MDSCs was associated with a worse outcome [136]. NOS1 expression in melanoma metastases was inversely correlated with therapeutic outcome to adoptive T cell therapy. However, in these patients, NOS2 and NOS3 expression in the melanoma tumors did not correlate with therapeutic outcome [68]. Although one study did not assess for overall survival, another study suggested that levels of nitric oxide increased in the peripheral blood of melanoma patients undergoing interferon- α therapy [137]. Whereas iNOS expression

levels were associated with overall survival level in melanoma, the same is not true for single nucleotide polymorphisms (SNPs). When single nucleotide polymorphisms (SNPs) were examined, no SNPs of the iNOS gene were associated with development of cutaneous melanoma whereas SNPs of the nNOS gene were associated with a risk of having moles and sunburns [138]. Interestingly, the use of iNOS as a biomarker may not be limited to cutaneous melanoma as iNOS may also be prognostic in uveal melanoma [139]. There is currently a phase Ib/II trial undergoing using omaveloxolone (NRF2 activator) to test the hypothesis that decreased reactive nitrogen species production will result in improved survival in patients with metastatic melanoma who are resistant to anti-PD-1 antibody therapy () [140].

Melanogenesis and tumor formation is controlled by NO metabolism

The pigment melanin has carcinogenic effects in addition to its well-known protective effects [141, 142]. Peroxynitrite, formed from superoxide and NO intermediates after UV exposure, transfers energy to melanin via chemiexcitation and induces cyclobutane pyrimidine dimers in DNA via radiationless energy transfer [141]. When B16-F10 cells were treated with a NO scavenger [MHY966] and subsequently implanted into mice, the tumors were smaller and contained decreased melanin content [143]. Premalignant melanocytes may be transformed into melanoma cells by anchorage blockade and promoting a state of eNOS uncoupling where eNOS preferentially produces high levels of superoxide to promote malignant transformation of a normal melanocyte cell line [144, 145]. This study reveals that it is not only the expression level of the NOS isoforms that is important but it is equally important to determine the nature of the free radical products that are generated. Other pathways may be involved in subsets of melanoma. For example in the BRAF mutated A375 murine models increased NO production increased expression microsomal prostaglandin E2 PGE2 synthase-1 and resulted in increased tumor growth [146]. It is important to remember that UVA mobilizes NO from skin stores to the systemic circulation where it lowers blood pressure [147]. Therefore, when formulating potential therapeutic interventions to limit tumor formation we must weigh the harmful effects of UV radiation versus potential benefits.

Conclusion

Although NO is primarily understood to be a gaseous signaling molecule, the evidence illustrates that NO has a multifaceted role in the development of melanoma. NO mediates effects through the melanoma cell itself, immune cell subsets (e.g. MDSC, DC, cytotoxic T cells, tumor associated macrophages), and the tumor microenvironment (e.g. vasculature, lymphatics). A dichotomy in this paper has been established in that NO can have both tumor promoting and inhibitory effects. Many of these effects are both concentration and context dependent. Further investigation to elucidate these individual pathways of NO modulation of melanoma progression can lead to possible new therapeutic strategies for melanoma.

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Abbreviations

AMG	Aminoguanidine
APE/Ref-1	Apurinic/aprimidinic endonuclease-1/redox factor –1
ARG1	Arginine 1
BCL2	B-cell lymphoma 2 gene
BRAF	B- Rapidly Accelerated Fibrosarcoma
cGMP	Cyclic guanosine monophosphate
eNOS	constititional nitric oxide synthase
COX-2	cyclooxygenase-2
c-PITO	2–4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CTL	cytotoxic T lymphocytes
CXCL10	chemokine (C-X-C motif) ligand 10
DAF-FM	4-Amino-5-methylamino-2',7'-difluorofluorescein (Diaminofluorescein)
DC-HIL	dendritic cell-associated, heparan sulfate proteoglycans-dependent integrin ligand
DR4	antigen D related 4
eNOS	endothelial NOS
GM-CSF	Granulocyte macrophage colony stimulating factor
HIF-1	hypoxia inducible factor-1
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
iNOS	inducible NOS
JAK/STAT	Janus kinase/Signal Transducer and Activator of Transcription proteins
JunD	JunD is the transcription factor protein product of the JUND gene
L-NAME	L-NG-Nitroarginine methyl ester

L-NIL	L-N6-(1-Iminoethyl)lysine
L-NMMA	NG-monomethyl L-arginine
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MDSC	Myeloid derived stem cells
MHY966	2-bromo-4-(5-chloro-benzol[d]thiazol-2-yl
MMP-1	Metalloproteinase-1
NAPDH	Nicotinamide adenine dinucleotide phosphate (Reduced)
nNOS	neuronal NOS
NO	Nitric oxide
NONOate	diethylaminedinitric oxide
NOS	Nitric oxide synthase
NOS1	neuronal NOS
NOS2	inducible NOS
NOS3	endothelial NOS
ONOO	Peroxynitrite
PAPA NO	(Z)-1- [N-(3-ammonio-propyl)-N-(n-propyl)amino]diazene-1-ium-1,2diolate
PDE-5	phosphodiesterase type 5
PD-L1	Programmed cell death ligand 1
pO₂	Partial pressure of oxygen
pSTAT1	phosphorylated Signal transducer and activator of transcription 1 (STAT1)
RET	rearranged during transfection gene
ROS	reactive oxygen species
SNAP	s-nitroso-n-acetyl-dl-penicillamine
SNP	Single nucleotide polymorphisms
TAM	tumor associated macrophage
TGFβ1	Transforming growth factor beta 1
VCAM-1	Vascular cellular adhesion molecule-1

VEGF

Vascular endothelial growth factor

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Figure 1. NO biochemistry.

NO is produced by various biochemical processes and is involved in posttranslational modifications of proteins.

Figure 2. NO general cellular function.

NO is involved in cGMP dependent and independent cell processes. It is involved in regulating mitochondrial function, apoptotic processes and multiple other pathways such as interferon response pathways and production of chemokines/cytokines. This figure has been generated utilizing the tools provided in the ChemDraw program (PerkinElmer, Waltham, MA, USA).

Figure 3. Physiologic roles of NO.

NO is important in regulating the activities of immune cells, metastatic spread to organs such as lung and liver, and the metabolic activity of melanoma cells themselves. This figure has been generated utilizing the tools provided in the ChemDraw program (PerkinElmer, Waltham, MA, USA).