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Inducible nitric oxide synthase (iNOS) drives mTOR pathway activation and proliferation of human melanoma by reversible nitrosylation of TSC2

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Abstract

Melanoma is one of the cancers of fastest-rising incidence in the world. iNOS is overexpressed in melanoma and other cancers, and previous data suggest that iNOS and nitric oxide (NO) drive survival and proliferation of human melanoma cells. However, specific mechanisms through which this occurs are poorly defined. One candidate is the PI3K/AKT/mTOR pathway, which plays a major role in proliferation, angiogenesis, and metastasis of melanoma and other cancers. We used the chick embryo chorioallantoic membrane (CAM) assay to test the hypothesis that melanoma growth is regulated by iNOS-dependent mTOR pathway activation. Both pharmacologic inhibition and siRNA-mediated gene silencing of iNOS suppressed melanoma proliferation and *in vivo* growth on the CAM in human melanoma models. This was associated with strong downregulation of mTOR pathway activation by Western blot analysis of p-mTOR, p-P70S6K, p-S6RP, and p-4EBP1. iNOS expression and NO were associated with reversible nitrosylation of TSC2, and inhibited dimerization of TSC2 with its inhibitory partner TSC1, enhancing GTPase activity of its target Rheb, a critical activator of mTOR signaling. Immunohistochemical analysis of tumor specimens from stage III melanoma patients showed a significant correlation between iNOS expression levels and expression of mTOR pathway

members. Exogenously-supplied NO was also sufficient to reverse mTOR pathway inhibition by the B-Raf inhibitor Vemurafenib. In summary, covalent modification of TSC2 by iNOS-derived NO is associated with impaired TSC2/TSC1 dimerization, mTOR pathway activation, and proliferation of human melanoma. This model is consistent with the known association of iNOS overexpression and poor prognosis in melanoma and other cancers.

Keywords

nitric oxide; mTOR; melanoma; nitrosylation; inducible nitric oxide synthase

INTRODUCTION

Melanoma incidence is increasing rapidly, and while surgical removal of early-stage disease is often curative, the mean survival time following metastasis is rarely beyond one year due to chemotherapeutically resistant disease (1). Thus it is critical to identify new molecular targets for melanoma therapy.

Inducible nitric oxide (NO) synthase (iNOS), is constitutively overexpressed in many cancers, including melanoma and gastric (2) breast (3) and head and neck (4) carcinomas. In melanoma, iNOS expression correlates strongly with poor clinical (5, 6). On balance, preclinical data support a pro-tumor role for iNOS/NO (7) by a variety of mechanisms (8–10). In both *in vitro* and *in vivo* models, iNOS and NO have been variously shown to enhance carcinogenesis and tumor progression, stimulate angiogenesis, support tumor growth, and promote metastasis (11, 12). Thus there is substantial interest in the molecular mechanisms through which iNOS and NO enhance tumor growth and aggressive behavior.

The PI3K-AKT-mTOR oncogenic signaling pathway (13) is one potential target of iNOS/NO. mTOR is activated by the small guanosine triphosphatase (GTPase) Ras homolog enriched in brain (Rheb) (14, 15), which is the target of the GTPase-activating domain of the tuberous sclerosis complex 2 (TSC2) protein. TSC2 is a tumor suppressor gene which, when complexed with TSC1, inactivates Rheb and thus inhibits activation of mTOR and downstream pathway members such as p70 ribosomal S6 kinase (P70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1).

Limited experimental evidence supports the hypothesis that iNOS/NO-driven mTOR activation can contribute to oncogenic signaling. Some studies have suggested that NO can activate mTOR signaling in non-cancer model systems, including murine macrophages (16), and vascular smooth muscle cells (17). However, as yet there have been no studies assessing the ability of cancer-expressed iNOS and endogenously-produced NO in physiologic concentrations to activate the mTOR pathway.

In the current study, we assessed the effect of iNOS inhibition with small-molecule antagonists or RNAi-mediated gene silencing (knockdown) on human melanoma in the chick chorioallantoic membrane (CAM) model, which has been widely used to study cancer growth and angiogenesis *in vivo* (18). Our data show that in human melanoma mTOR pathway activation is dependent on iNOS expression/activity, and support a model where

iNOS modulates mTOR activity by NO-mediated post-translational modification of the upstream regulator TSC-2.

MATERIALS AND METHODS

Tumor cell lines and chick embryo chorioallantoic membrane (CAM) model of tumor growth

Authenticated human melanoma lines A375 and MeWo were obtained from American Type Culture Collection (ATCC). The line mel624 was obtained from the laboratory of E. Grimm, UT MD Anderson Cancer Center, and authenticated by the MD Anderson Characterized Cell Lines Core Facility. For CAM assays, 2×10^6 log-phase, A375 cells were implanted in 25 μ L Matrigel® on the CAM of 10-day-old chick embryos (SPAFAS, North Frankling, CA) as described (19, 20). Implanted cells were treated with L-NIL applied topically onto the upper CAM, or transfected 24 hours earlier with iNOS-targeting, scrambled, or non-targeting control siRNA. After 6 days, tumor growth was evaluated macroscopically, and tumor tissue with a minimal amount of surrounding CAM was minced, dissociated into single cell suspension with type 1A collagenase, and live tumor cells counted by trypan blue exclusion. Alternatively, excised CAM tissues were homogenized with passive lysis buffer (Boston Bioproducts) for western blot analysis; or fixed in 10% zinc formalin, embedded in paraffin, cut in 5- μ m sections, and analyzed by hematoxylin and eosin staining and immunohistochemistry (IHC).

Transient transfection of human melanoma cells

A375, Mel624 and/or MeWo cells were transfected with Lipofectamine according to the manufacturer's (Invitrogen) protocol. At 24 h after transfection, cells were re-plated for use in the indicated assays. Plasmid expressing the full-length murine GFP- iNOS sequence was a generous gift from Ignacio Rodriguez-Crespo (Complutense University of Madrid, Madrid, Spain) and has been previously described (21). pMSCV-puro GFP vector (Addgene, Cambridge, MA) was used as a control. Human iNOS-targeting siRNA Stealth RNA duplexes and non-targeting control siRNA (catalog number 46-2002) were from Invitrogen (Grand Island, NY, USA).

Chemicals and reagents

L-NIL (hydrochloride) and DEANOONOate were purchased from Cayman Chemical (Ann Harbor, MI). PTIO, SNAP, and Dithiothreitol were from Sigma-Aldrich (St. Louis, MO). Vemurafenib (PLX4032/RG7204) from Selleckchem (Houston, TX). All chemicals were prepared freshly from powdered stock on the day of each experiment. All cell culture reagents were from Invitrogen.

Western blots

Following SDS-PAGE and transfer to nitrocellulose, membranes were incubated overnight at 4°C with primary antibodies against iNOS (sc-651, Santa Cruz Biotech), mTOR (sc-2983 Santa Cruz), p-mTOR Ser 2448 (sc-2971 Santa Cruz), Phospho-4E-BP1 (Thr37/46) (sc-9459, Santa Cruz), 4E-BP1 (sc-9977, Santa Cruz), Tuberin/TSC2 (D57A9) (sc-3990, Santa Cruz), Hamartin/TSC1 (sc-4906), Actin (sc-1616, Santa Cruz) followed by

appropriate secondary antibody conjugated to horseradish peroxidase. Results were visualized by commercial chemiluminescence kit (Millipore). Relative signal intensities for total and phospho-proteins were quantitated by densitometry using Image-J software (National Institutes of Health, Bethesda, MD), and normalized densitometry values were expressed as ratio of phospho- to total protein levels.

Immunoprecipitation

Cells were seeded into 6-well plates and incubated for 1 hour at 37°C with 100 µM or 300 µM fresh SNAP, 100 µM fresh DEANONOate, 1000 µM or 3000 µM L-NIL. Cells were washed with Ice-cold PBS and lysed in NP-40 and X100 plus protease inhibitors for 10 minutes on ice, then cleared by centrifugation (10000 g for 10 Minutes at 4°C). For TSC2 immunoprecipitation the antibody (Bethyl Laboratories cat A300-526A) was incubated with lysates on ice for 90 minutes before treatment with protein A beads and incubation at 4°C for 3 hours with gentle rotation. Beads were washed, recovered between each wash by centrifugation (1000 g for 30 seconds at 4 °C), and resuspended in sample buffer prior to immunoblot analysis.

Immunohistochemistry of CAM and clinical melanoma specimens

Formalin-fixed, paraffin-embedded (FFPE) sections (5µM) of excised human melanoma tumors from CAM were immunostained for Ki-67 (sc-23900 Santa Cruz), S100 (ab14849, Abcam), or HMB45 (Ventana Medical System, Inc ; Tucson, Arizona), p70 S6K (49D7) (Cell Signaling Cat # 2708), Phospho-4E-BP1 (Thr37/46) (236B4) (Cell Signaling Cat # 2855), 4E-BP1 (53H11) (Cell Signaling Cat # 9644), iNOS (Creative Biolabs, Shirley, NY), nitrotyrosine (NT) (EMD Millipore Corp., Billerica MA). Pre-immune normal mouse IgG (Vector Laboratories, Burlingame, CA) and anti-vimentin antibody (BioGenex Laboratories, San Ramon, CA) were used as negative and positive controls, respectively

For Ki-67 staining of CAM tissue, the fractional area occupied by the immunoreactive ki-67 was quantified with the Bioquant image analysis system (BIOQUANT Image Analysis Corporation, Nashville, TN) as previously described (22). Random fields from seven independent sections were analyzed per sample and averaged.

Rheb GTP γ S Guanine Nucleotide Binding Assay

A375 cells were transfected with iNOS-targeting or control siRNA as described above. Forty hours after transfection, cells were washed once with phosphate-free Dulbecco's modified Eagle's Medium (Invitrogen), and processed with the Rheb Activation Assay Kit (NewEast Biosciences, Malvern, PA) as previously described (23). For positive and negative controls, A375 cell lysate, was incubated with guanosine 5'-O-[γ -thio] triphosphate (GTP- γ S) or GDP respectively for 90 min before immunoprecipitation.

Biotin switch assay of S-Nitrosylation

The biotin switch assay was performed according to the method of Jaffrey and Snyder (24) with some modifications. Cell lysates were prepared in SDS sample buffer incubated for 16 hours at 37C in the dark with either 100 µM fresh SNAP, 1000 µM L-NIL, 300 µM PTIO, and 1 hour with 10 mM DTT room temperature or left untreated, after which they were

subjected to biotin-switch assay using the S- Nitrosylated Protein Detection Kit (Cayman Chemical). After the biotin-switch assay, Biotinylated proteins were pull-down with streptavidine-agarose beads (Thermo Scientific) and the eluate was resolved by SDS-PAGE and analyzed by immunoblotting.

Clonogenic survival assay

Cells were seeded into 6-well plates at concentrations of 500/ well or 1000/well in triplicate. After 24 hours, L-NIL was added or cells were transiently transfected with siRNA as described above before changing to serum-containing medium. Cells were incubated for 10-14 days to form visible colonies. Colonies were stained with methylene blue, rinsed with water, air-dried and imaged and quantified by using ImageJ, and colony number was counted. Cell colonies were visually identified and colonies greater than 50 cells counted (25)

TUNEL staining

FFPE tissue sections (5 µm) from CAM were processed for terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL). The ApoTag Fluorescein in Situ Apoptosis detection Kit (Millipore, Billerica, MA) was used according to the manufacturer's instruction.. 5 different fields were counted per sample. TUNEL kit : S7110, EMD Millipore Corporation, Billerica, MA. Mounting Medium with Propidium Iodide is: H-1300, (Vector Laboratories, Burlingame, CA).

Analysis of cell cycle progression

Cells were plated at a density of 1×10^5 cells/5ml medium in 60 cm-diameter dishes and L-NIL added 24 hours later in fresh medium. Adherent cells were harvested 48 hours later by trypsinization and centrifugation. After washing with PBS, propidium iodide was added and the DNA content of stained nuclei analyzed by flow cytometry with the FACSCalibur using Flowjo 7.6 software.

Statistical methods

Associations between staining number and intensity levels of iNOS, nitrotyrosine, p-4EBP1, and p70S6K co-expression in clinical melanoma samples were assessed using Fisher's exact tests. Overall survival was determined from stage III diagnosis to last known vital status. Patients alive at the last follow-up date were censored. Associations between iNOS expression levels and overall survival were assessed using univariate Cox proportional hazards models. Throughout the paper: error bars indicate standard error of the mean; * indicates $p < 0.05$ significance; ** indicates $p < 0.01$ significance

RESULTS

iNOS inhibition or knockdown suppress *in vivo* growth of human melanoma in the CAM model

Nitrotyrosine is a stable NO end product and marker of cumulative iNOS activity (26). To assess the contribution of iNOS to human melanoma growth in a physiologically relevant

setting, we established primary A375 melanoma tumors in the CAM assay and treated them with the iNOS selective competitive antagonist L-N-(1-Iminoethyl) Lysine (L-NIL) (27, 28) or gene knockdown with iNOS-targeting small interfering RNA (siRNAs) (29) iNOS knockdown decreased constitutive iNOS gene expression (Fig 1A), and both siRNA and L-NIL markedly suppressed nitrotyrosine accumulation in A375 tumors grown on CAM (Fig 1A, B).

Well-vascularized 3-dimensional tumor nodules were present by 6 days after inoculation of melanoma cells into matrigel upon the CAM. Immunohistochemical staining with the melanoma markers S100 (Fig. 2A) (30), and HMB45 (data not shown) as well as conventional H&E sections (Fig. 2B) verified discrete human melanoma tumors on the background of chicken-derived CAM cells. iNOS knockdown (Fig. 2C) or L-NIL (Fig. 2D) suppressed both macroscopic tumor growth across several human melanoma cell lines (A375, Mel624 and MeWo), and the number of viable tumor cells (Fig. 2C-B, S1A,B).

Growth suppression of human melanoma after iNOS knockdown or inhibition is associated with decreased clonogenic survival and proliferation, and increased apoptosis

The clonogenic survival assay has been shown to correlate with tumor cell proliferative capacity *in vivo* (31). Both iNOS inhibition and knockdown suppressed *in vitro* clonogenic growth of A375 cells (Fig. 3A), consistent with the marked reduction in *in vivo* tumor growth. L-NIL treatment *in vitro* also arrested A375 cells at the G₂/M phase of the cell cycle (Fig. 3B), (32). iNOS knockdown *in vivo* also inhibited by over 50% immunostaining for the proliferation marker Ki-67 (Fig. 3C) (33). Furthermore, iNOS knockdown increased by two-fold apoptosis *in vivo* as measured by TUNEL assay (Fig. 3D) (34). Thus, the marked melanoma growth suppression observed with iNOS inhibition or silencing is the result of concomitant inhibition of proliferation and increased apoptotic cell death.

Constitutive activation of the mTOR growth/survival pathway *in vivo* is downregulated by knockdown or inhibition of iNOS in human melanoma

Since the mTOR pathway has been shown to be a key mediator of proliferation and survival for melanoma (35) and other other tumors (36) we focused on this pathway in two human metastatic melanoma cell lines grown in CAM, A375 (bearing the clinically-significant BRAFV600E mutation; Fig. 4A, B), and MeWo (wild-type BRAF; Fig. 4C, D). iNOS-targeting siRNA and iNOS inhibition with L-NIL coordinately downregulated expression of the activated (phosphorylated) form of multiple mTOR pathway members in a dose-dependent fashion (Fig. 4A,B).

Specificity of the iNOS-targeting siRNA is demonstrated by the similar effect of siRNA constructs targeting different regions of the iNOS gene (Fig. 4C), and the lack of effect of two different control constructs derived from scrambled iNOS-targeting sequences (Fig. 2C, 4A,C) We further confirmed specificity of iNOS-targeting siRNA by performing genetic rescue of siRNA-treated cells with a construct expressing GFP fused to murine iNOS (GFP-iNOS; Fig. 5A) (21), which is incompletely homologous with the human ortholog and thus resistant to knockdown (Fig. S2). GFP iNOS markedly upregulated iNOS expression and reversed siRNA-mediated inhibition of S6RP and 4EBP1 phosphorylation (Fig. 5B). We

also noted modest, but reproducible upregulation of S6RP and 4EBP1 phosphorylation in cells transfected with the GFP-iNOS construct but not GFP alone, consistent with previously published data (37). Taken together, our results demonstrate that endogenous iNOS expression is required to maintain high levels of constitutive mTOR pathway activation in two human melanoma and that inhibition of iNOS activity or gene expression markedly downregulates mTOR signaling in melanoma cells in a reversible fashion.

iNOS-mediated S-Nitrosylation of TSC2 regulates TSC2/TSC1 dimerization and the activity of the mTOR-activating small GTPase Rheb

S-Nitrosylation is a reversible NO-mediated post-translational modification of cysteine residues which can affect protein stability or activity, and thus a candidate mediator of activation by iNOS. To examine whether this mechanism occurs in human melanoma cells, A375 cells were treated with S-nitroso-penicillamine (SNAP, a donor of NO); 2-(4-Carboxyphenyl)-4,4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO, an NO scavenger); or L-NIL for 16 hours before harvest of cell lysates and biotin switch assay to detect nitrosylated proteins (38). Constitutively nitrosylated proteins biotinylated during the biotin switch reaction were observed in untreated and SNAP-treated lanes, with a marked reduction in biotinylation intensity in lysates from L-NIL and PTIO treated cells (Fig. 6A), demonstrating reversible nitrosylation of endogenous proteins in melanoma.

To assess nitrosylation of specific mTOR pathway proteins we performed a streptavidin-agarose pulldown after the biotin switch reaction, followed by immunoblot for specific proteins of interest. This method successfully detected nitrosylation of several control proteins previously known to be S-nitrosylated, including Bcl-2 (39) and AKT (40) (Fig. S3). We observed endogenous nitrosylation of TSC2, which was enhanced by treatment of the cell lysate with the NO donor SNAP. Reversibility of TSC2 nitrosylation was confirmed by loss of the SNO-TSC2 band after biotin switch with lysate from L-NIL- or PTIO-treated cells (Fig. 6B). However, total TSC2 levels were not affected, suggesting that nitrosylation does not affect TSC2 stability.

TSC1/TSC2 dimerization is a critical checkpoint for downstream Rheb activation required for mTOR pathway signaling. To further characterize the mechanism of NO-mediated control of TSC2 activity, we performed co-immunoprecipitation studies to determine the effect of NO on TSC1/TSC2 binding. A375 cells were treated with 2 structurally different NO donors DEANONOate and SNAP (Fig. 6C) or L-NIL. We immunoprecipitated (IP) cell lysates with anti-TSC2 antibody and performed immunoblotting of immunoprecipitate complexes (IP) and crude lysates (total lysate) with anti-TSC1, - TSC2, or -actin antibody. NO treatment decreased the binding of TSC1 to TSC2, whereas L-NIL enhanced binding. These experiments demonstrate that iNOS/NO modulate dimerization of TSC2 with its inhibitory partner TSC1. We next investigated the association of TSC2 nitrosylation with activation of downstream signaling, by assessing Rheb GTPase activity. iNOS-targeting siRNA almost completely inhibited Rheb activation (Fig. 6D), demonstrating control of Rheb activity by iNOS. While iNOS and NO can potentially also control mTOR pathway activation by acting on AKT or on upstream pathways which modulate AKT activation (41), we saw no evidence that iNOS inhibition downregulated AKT phosphorylation

(supplemental figure S4). We also saw little effect of the NO-dependent guanyl cyclase inhibitor ODQ, on mTOR pathway activation (supplemental figure S5), suggesting that iNOS/NO regulation is independent of this mechanism. Thus, we infer that iNOS/NO indirectly control Rheb activity by nitrosylation of the immediately upstream regulator TSC2.

Co-expression of iNOS, Nitrotyrosine, p-4EBP1, and p70S6K in clinical melanoma samples, and effect of NO on mTOR signaling downstream of B-Raf

We used immunohistochemistry to examine expression of iNOS, NT, p-4EBP1, and p70S6K *in vivo* in tissue microarrays (TMA) from metastatic tumors from stage III melanoma patients (representative stains shown in Fig. 7A). Staining for iNOS was discrete and variable among tumors, while the NO biomarker NT showed more diffuse staining, consistent with the high diffusivity of NO. Antibodies to phospho-4EBP1 and p70S6K also showed the anticipated cytoplasmic staining.

To explore whether iNOS and NT staining levels correlated with mTOR pathway activation, we evaluated staining number (% of cells positively staining) and intensity for all markers in random fields from 3 TMA cores (6) and tested for pairwise associations between markers, and between staining intensity and overall and disease-free survival. Of 118 available patient samples, staining data was available for 112 (NT), 108 (iNOS), 103 (p-4EBP1), and 100 (p70S6K) patients. We found a significant correlation between iNOS and NT staining number ($p = 0.0043$) and intensity ($p < 0.0001$), consistent with the role of iNOS as the primary NO-producing enzyme in human melanoma cells. We also found statistically significant associations between phospho-4EBP1 and p70S6K number ($p < 0.0001$) and intensity ($p < 0.0001$) by Fisher's exact test, presumably reflecting upregulation of both markers during mTOR pathway activation. iNOS staining intensity and NT staining intensity were significantly correlated with p70S6K and NT intensity, respectively (Fig. 7B). Thus we observed a positive association between iNOS upregulation and mTOR pathway activation in clinical melanoma specimens similar to what we observed in human melanoma in the CAM assay

We then examined the effect of NO on mTOR signaling downstream of the BRAF inhibitor Vemurafenib (PLX4032/RG7204) (42, 43) since it is used clinically in treatment of melanoma. Cells were treated with Vemurafenib and the exogenous NO donor DEANOONOate, or a combination of both for 3 hours. Western blot analysis revealed that short-term treatment with Vemurafenib alone markedly reduced phosphorylation of downstream mTOR pathway members S6RP and 4EBP1 in A375 lysates. We found that exogenously-supplied NO reverses mTOR inhibition seen after Vemurafenib treatment (Fig. 7C, D), suggesting that NO can act downstream of BRAF to bypass BRAF inhibition, and thus may serve as a potential mechanism of escape of inhibition.

DISCUSSION

In the present study we used human melanoma grown in the *in vivo* CAM model to test the hypothesis that iNOS upregulation by cancer cells enhances tumor growth and survival by driving mTOR pathway activation, a critical mediator of oncogenic signaling. We found that

iNOS expression in human melanoma leads to nitrosylation of TSC2 which is associated with impaired dimerization of TSC2 with its inhibitory partner TSC1 and enhanced activation of its target, the small GTPase Rheb, and subsequent activation of downstream members of the mTOR pathway. iNOS-mediated activation of mTOR signaling is oncologically significant, leading to enhanced proliferation as measured by Ki-67 immunostaining and cell cycle analysis, decreased apoptosis, and macroscopic tumor growth on CAM. We also find evidence that iNOS-driven mTOR activation is clinically relevant, since iNOS and nitrotyrosine expression are positively correlated with S6K and phospho-4EBP1 upregulation by immunohistochemistry of metastatic human melanoma samples.

In the present study we functionally dissect the role of endogenous iNOS/NO expression with the selective iNOS inhibitor L-NIL and iNOS-targeting siRNA, thus showing that the upregulation of iNOS previously reported in melanoma and many other cancers is itself sufficient to drive mTOR pathway activation and tumor growth. This is consistent with prior studies showing that iNOS upregulation induces many of the hallmarks of mTOR activation (proliferation, survival, angiogenesis, treatment resistance) and is associated with poor prognosis in human melanoma patients. In fact, downregulation of mTOR activation by iNOS inhibition or knockdown was associated with a striking reduction in melanoma growth on the CAM, similar to that which we have previously reported in mouse xenograft studies (27). We also noted a robust effect of iNOS inhibition or knockdown on *in vitro* colony formation by human melanoma cells, suggesting that even *in vitro* iNOS may be required for optimum clonogenicity of founder cells. We also found that exogenously-supplied NO reverses mTOR pathway inhibition seen after Vemurafenib treatment, suggesting that NO can act downstream of BRAF to bypass BRAF inhibition, and thus is a potential mechanism of escape (44, 45)

While NO is pleiotropic, and can potentially regulate mTOR pathway activation through other mechanism, such as nitrosylation of EGFR and other molecules upstream of mTOR signaling (PMID 22878588) our study is also the first to propose nitrosylation of an mTOR pathway member as a biochemical mechanism linking cancer-related iNOS/NO overexpression with pathway activation. NO can alter signal transduction pathways via covalent modification of target proteins through nitration and nitrosylation. We show by biotin switch assay that TSC2, which in the activated state acts to suppress mTOR activation, by forming a Rheb-inactivating complex with TSC1, is constitutively nitrosylated in melanoma cells. Immunoprecipitation experiments demonstrate that iNOS/NO modulates dimerization of TSC2 with its inhibitory partner TSC1, a critical checkpoint for downstream Rheb activation; thus modulating physical association of TSC1 and TSC2 is a plausible and novel mechanism by which iNOS/NO can control mTOR pathway activation. TSC2 has many cysteines in functionally-relevant areas of the protein which could potentially be nitrosylated, and ongoing work in our laboratory is focused on identifying specific cysteines which are targets for nitrosylation in melanoma cells.

A limitation of our study approach is the possibility that iNOS is abnormally dysregulated in the CAM system, and thus our results are not relevant to melanoma in human patients. However, melanoma lines used in this study constitutively express iNOS even in tissue

culture, and iNOS expression is well-documented in human melanoma cell lines and clinical specimens. Also the correlation between iNOS and NO expression levels and S6K and 4EBP1 we observed in human clinical metastatic melanoma specimens supports the relevance of this mechanism to clinical melanoma. While other NOS isoforms (NOS1, NOS3) may also be expressed in cancer cells and play a role in their biology, we conclude that in our system iNOS is a key driver of NO-mediated signaling in human melanoma cells.

iNOS and other inflammatory molecules have been shown to play pivotal roles in carcinogenesis, tumor progression, proliferation and apoptosis resistance of human cancers. Molecular therapy targeting cancer-related inflammation is an attractive approach to cancer treatment, since iNOS and other inflammatory mediators are broadly overexpressed in a number of human cancers, largely dispensable for untransformed cells, and amenable to pharmacologic inhibition. We and others have described potent anti-tumor effects of iNOS inhibition which are both direct (46, 47) and indirect (48) highlighting the dual role of iNOS as signaling molecule and inflammatory mediator. Our results provide a convincing biological mechanism by which iNOS upregulation activates oncogenic signaling in melanoma cells through the post-translational modification of TSC2, and set the stage for further investigation into specific mechanisms linking inflammation, redox signaling, and cancer biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. iNOS Knockdown

B. iNOS Inhibition

Figure 1. Effect of the iNOS-selective competitive antagonist L-NIL or iNOS siRNA knockdown on iNOS protein and nitrotyrosine levels

A375 cells were (A) transfected with siRNA against iNOS or control siRNA at the indicated concentration and cultured for 24 hours before inoculation on the CAM or (B) or allowed to grow on CAM in the presence of the indicated concentrations of L-NIL. After 5 days, tumors were excised and whole cell lysates subjected to SDS-PAGE and western blot for iNOS, nitrotyrosine and actin. Representative western blots are shown, and the

accompanying graphs show relative nitrotyrosine (NT) levels derived from 3 independent experiments.

Figure 2. iNOS knockdown or targeted inhibition inhibits *in vivo* melanoma growth in the CAM model

A375 cells were inoculated, grown, and harvested on the CAM as described in Figure 1. (A) Formalin-fixed, paraffin-embedded (FFPE) tissue was subjected to immunostaining for the melanoma marker S100, demonstrating S100-positive melanoma cells against a background of unstained CAM. (B) Control and L-NIL (1000 μM)-treated tumors were stained with hematoxylin-eosin (H&E). (C) Photography of macroscopic tumors and associated vasculature of A375 cells transfected with 100 nM scrambled control or iNOS-specific

siRNA. Tumor areas are outlined with black dashed lines (for scale, teflon ring is 5 mm). To the right is quantiation (from 3 experiments) of tumor cells by trypan blue exclusion assay .

(D) In a parallel experiment, A375 cells were treated with the indicated doses of L-NIL before growth on CAM, photography and quantitation.

Figure 3. iNOS knockdown or targeted inhibition causes G2/M phase cell cycle arrest and decreases clonogenic survival *in vitro*, and promotes apoptosis *in vivo*

(A) A375 cells were transfected with control (Ctrl) or iNOS-targeting siRNA or the indicated concentration of L-NIL (300 and 1000 μ M) and subjected to clonogenic growth assay. Representative images are shown for L-NIL. Graphs indicate the number of colonies, relative to control. Data are averaged triplicate values of one independent experiment (representative of 3). (B) Flow cytometric analysis of cell cycle distribution of propidium iodide (PI) treated melanoma cells after 48 hours L-NIL treatment. Representative

histograms are shown; bar graphs show pooled data from 2 experiments FL2-Area (FL2-A). (C–D) A375 cells were inoculated on the CAM after transfection with control (scramble 100 nM) or iNOS-targeting siRNA (100 nM) as described in Figure 1 and allowed to grow for 6 days before harvest of the tumor and associated CAM for histological processing and Ki-67 immunostaining (C) or TUNEL analysis (D). For TUNEL the number of TRITC-labeled green apoptotic cells in a minimum of 5 fields per experiment are expressed as the ratio of TUNEL-positive cells to the total number of PI-labeled (red)nuclei. For both Ki67 and TUNEL staining, quantitative imaging analysis was performed with BIOQUANT image analysis software; bar graphs show the relative number of TUNEL+ or Ki-67+ cells averaged over 3 independent experiments.

Figure 4. iNOS knockdown or targeted inhibition suppresses mTOR pathway activation *in vivo*
A375 cells were transfected with (A) iNOS-targeting or control siRNA or (B) the indicated concentrations of L-NIL and CAM assay performed as described in Figure 1. Whole cell lysates were subjected to SDS-PAGE and western blot analysis for mTOR phosphorylated at serine 2448 (Ser2448), p-P70S6K phosphorylated at threonine 389 (Thr389), p-4EBP1 phosphorylated at threonine 37/46 (Thr37/46), p-S6RP phospho at serine 235/236 (Ser235/236), and actin as a loading control. (C) MeWo cells were transfected with iNOS-targeting or control siRNA before CAM assay and western blot for mTOR, p-P70S6K, p-4EBP1, p-S6RP, and actin. (D) Quantitation of phospho-4EBP1 versus total protein and phospho-S6RP versus actin were by densitometric analysis. All graphs represent data derived from 3 independent experiments.

Figure 5. Overexpression of murine iNOS reverses the effect of iNOS knockdown on mTOR pathway activation

(A) A375 Cells were transfected with GFP or murine GFP-iNOS constructs and imaged 48 hours later by fluorescence microscopy. Note that only iNOS-GFP-transfected cells display aggresomes (arrowhead). (B) To rescue the effects of iNOS knockdown, cells were transfected with iNOS siRNA, Scramble siRNA, murine GFP-iNOS, or GFP constructs alone or in combination before inoculation on the CAM as described above. Western blot analysis was performed to confirm the knockdown of endogenous iNOS and overexpression

of murine GFP-iNOS; rescue of mTOR pathway activation by mouse GFP-iNOS was assayed by Western blot for the downstream pathway member S6RP. Actin was used as the loading control. One representative blot of 3 is shown. The bar graphs show the relative expression level (normalized to actin).

Figure 6. Reversible S-nitrosylation of TSC2 blocks TSC1-TSC2 dimerization and upregulates Rheb activity

A375 cells were cultured with the indicated concentrations of L-NIL, the NO donor S-nitroso-acetyl-penicillamine (SNAP), or the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3 oxide (PTIO) for 16 Hours prior to harvest and preparation of protein lysates. As a negative control, parallel lysates were exposed to 10 mM of SH-reducing agent dithiothreitol (DTT) for 1 hour *in vitro*. (A) Total S-nitrosylated protein levels were determined by biotin switch assay and anti-biotin immunoblot; “unbiotinylated”

shows endogenous biotin levels. **(B)** S-Nitrosylation of TSC2 in biotin switch assay followed by immunoprecipitation with streptavidine agarose and immunoblot analysis with anti-TSC2 polyclonal antibody. “SNO” (S-nitrosothiol) indicates biotinylated (nitrosylated) TSC2 levels following biotin switch. “Total” indicates total TSC2 levels prior to immunoprecipitation. Actin was used as a loading control. **(C)** Effect of iNOS/NO on TSC2/TSC1 dimerization. A375 cells were treated with NO donors DEANONOate or SNAP, or the selective iNOS inhibitor L-NIL, and cell lysates were immunoprecipitated (IP) with anti-TSC2 antibody. Immunocomplexes (upper panel) and crude lysates (lower panel) were immunoblotted with anti-TSC1, -TSC2, or α -actin antibody. The ratio of TSC1 to TSC2 was calculated and normalized against the amount of TSC2 present after the immunoprecipitation **(D)** Effect of iNOS/NO on Rheb activation. A375 cells were transfected with iNOS-targeting siRNA or scrambled control and cultured for 48 hours. Western blots for activated Rheb (Rheb/GTP) total Rheb, including the inactive GDP-Bound form (GDP), and actin loading control were performed. Activated Rheb was quantified by densitometry and normalized to total Rheb levels. All graphs represent data from 3 independent experiments.

Figure 7. iNOS/NO upregulation is correlated with mTOR pathway activation/expression in regional metastases of stage III melanoma patients; NO reverses mTOR pathway inhibition by the B-raf inhibitor Vemurafenib

(A) Formalin-fixed paraffin-embedded (FFPE) tissue sections of tumor samples were stained for iNOS, nitrotyrosine (NT), phospho-4EBP1, or p70S6K expression by IHC. Pre-immune normal mouse IgG and anti-vimentin antibody were used as negative and positive controls, respectively. Hematoxylin was applied as a counter stain. 20× magnification. (B) Immunolabeling was scored separately for 2 variables: first, for number of positive melanoma cells; second, for the overall staining intensity of positive cells as described in the

materials and methods, with 0 being the lowest composite score, and 3 the highest. The slides were independently interpreted by 2 readers without knowledge of the clinical data. (C) Effects of the BRAF inhibitor Vemurafenib (VEMU) and NO donor DEANONOATE (DEA) alone and in combination on mTOR pathway activation. A375 melanoma cells were exposed for 3 hours to 3 μ M Vemurafenib, 100 μ M of the donor DEANONOate, or the combination. mTOR pathway activation was analyzed by 4EBP1 and p-S6RP western blot. Signals were quantified by densitometry, and the ratio of activated p-4EBP1 and p-S6RP was calculated, normalized against total protein (right panel). All graphs represent data from 3 independent experiments.