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Original Contribution

Endothelial nitric oxide synthase uncoupling as a key mediator of melanocyte malignant transformation associated with sustained stress conditions

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ABSTRACT

Melanoma cell lines and cells corresponding to premalignant melanocytes were established by our group after subjecting a nontumorigenic murine melanocyte lineage, melan-a, to sequential cycles of anchorage blockade. Previous results showed that in melan-a cells the superoxide level increases after such procedure. Superoxide production during melanocyte de-adhesion was inhibited by L-sepiapterin, the precursor of eNOS cofactor BH_4, and increased by the inhibitor of BH_4 synthesis, DAHP, hence indicating a partial uncoupling state of eNOS. The eNOS uncoupling seems to be maintained in cells derived from melan-a, because they present decreased nitric oxide and increased superoxide levels. The inhibition of superoxide production in Tm5 melanoma cells with L-sepiapterin reinforces their eNOS-uncoupled state. The maintenance of oxidative stress seems to be important in melanoma apoptosis resistance because Mn(III)TBAP, a superoxide scavenger, or L-sepiapterin renders Tm5 cells more sensitive to anoikis and chemotherapy. More importantly, eNOS uncoupling seems to play a pivotal role in melanocyte malignant transformation induced by sustained anchorage impediment, because no malignant transformation was observed when L-NAME-treated melanocytes were subjected to sequential cycles of de-adhesion. Our results show that uncoupled eNOS contributes to superoxide production during melanocyte anchorage impediment, contributing to anoikis resistance and malignant transformation.

Reactive oxygen species (ROS) are natural aerobic metabolic products, and their generation correlates with activation of signaling pathways controlling biological processes such as cell proliferation, differentiation, and migration [1,2]. The effects of ROS depend on the balance between their generation and their detoxification by enzymatic or nonenzymatic systems [3]. Moderate increases in ROS levels can promote cell proliferation and differentiation, whereas excessive amounts of ROS can cause oxidative damage to lipids, proteins, and DNA [4]. Therefore, the maintenance of ROS homeostasis is crucial for normal cell growth and survival. Several observations have also suggested the involvement of ROS in malignant transformation and tumor progression [5,6]. ROS production is increased in some tumor types, including melanoma, a finding that has been associated with mitogenesis, apoptosis resistance, and angiogenesis [7,8]. Mitochondria are the main source of ROS in nonphagocytic cells, but other cytosolic enzymatic systems, such NADPH oxidases, xanthine oxidases, and nitric oxide synthases, can also generate superoxide [9].

It has been demonstrated that uncoupled nitric oxide synthase (NOS) can also be a source of superoxide production [10,11]. NOSs are homodimeric oxidoreductases that catalyze NO production from L-arginine guanidine nitrogen using molecular oxygen. The NOS reductase domain shares a close homology with cytochrome P450 enzymes, generating electron flow from NADPH through FAD and FMN flavins. These are then transferred to the oxidase domain of other monomers in which L-arginine oxidation occurs at the heme group in the active site. Multiple integrated pathways, including activation by calcium–calmodulin, phosphorylation at key serine and threonine residues, and substrate and cofactor availability, regulate NOS activity [12]. A critical aspect of NOS function is the requirement of the cofactor tetrahydrobiopterin (BH_4). In its absence NOS dimerization is destabilized and NOS catalytic activity becomes "uncoupled,"

Abbreviations: PMA, phorbol 12-myristate 13-acetate; BH_4, tetrahydrobiopterin; BH_2, dihydrobiopterin; DAHP, 2,4-diamino-6-hydroxypyrimidine; L-NAME, L-N^G-nitroarginine methyl ester; ROS, reactive oxygen species; NOS, nitric oxide synthase; DAF-2DA, diaminofluorescein-2-diacetate; DHE, dihydroethidium; Mn(III)TBAP, Mn (III) tetrakis(4-benzoic acid) porphyrin; MnSOD, manganese superoxide dismutase; GTPCHI, GTP cyclohydrolase I; PTIO, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1*H*-imidazolyl-1-oxy-3-oxide; DNMT, DNA methyltransferase; VEGF, vascular endothelial growth factor; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

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resulting in superoxide formation [13]. NOS-dependent superoxide formation has a central role in the pathogenesis of vascular diseases such as diabetes, hypertension, and atherosclerosis [14,15].

At least to our knowledge, there is no information relating NOS uncoupling and malignant transformation and tumor progression. Many studies have shown increased expression of inducible (i) NOS in tumor cells, including melanomas, but no mechanisms have been described regarding the products generated by this enzyme [16]. Recently, an in vitro murine melanocyte malignant transformation model was developed in our laboratory, after subjecting a nontumorigenic melanocyte lineage, melan-a, to sequential cycles of anchorage impediment [17]. During anchorage blockade, melanocytes present increased levels of superoxide, nitric oxide, and hydrogen peroxide [18]. Surprisingly, L-N^G-nitroarginine methyl ester (L-NAME), a NOS inhibitor, abrogated the superoxide increase, but not nitric oxide levels, in melanocytes maintained in suspension. Additionally, the endothelial (e) NOS transcript level was also augmented under this condition. The aim of this study was to identify the sources of superoxide produced during melanocyte anchorage blockade and the impact of superoxide in melanocyte malignant transformation.

Experimental procedures

Cell culture

The nontumorigenic melan-a melanocyte lineage [19] was cultured at 37 °C in humidified 95% air–5% CO_2 in RPMI, pH 6.9, supplemented with 5% (v/v) fetal bovine serum (Invitrogen, Scotland, UK), 200 nM phorbol 12-myristate 13-acetate (PMA; Calbiochem, Darmstadt, Germany), 100 U/ml penicillin, and 100 U/ml streptomycin (Invitrogen, Grand Island, NY, USA). Premalignant melanocyte lineage 4 C, nonmetastatic melanoma cell line 4C11−, and metastatic melanoma cell lines 4C11+ and Tm5, established after subjecting melan-a cells to sequential cycles of anchorage blockade [17], were cultured as melan-a cells, but in the absence of PMA. For all experiments, cultures at 70 to 80% confluence were used, because cellular density influences ROS production.

De-adhesion cycles in the presence of L-NAME

The nontumorigenic melan-a lineage (10^5 cells/ml) was plated on 1% agarose-coated plates and cultured for 96 h as described above in the presence of 1 mM L-NAME (Cayman Chemical, Ann Arbor, MI, USA). Small spheroids were collected by decantation and plated under adherent conditions. Cells were allowed to proliferate to subconfluent growth. De-adhesion (spheroid formation) cycles were repeated four times; after the last de-adhesion step, spheroids were counted and plated at limiting dilution (0.5–1 spheroid/well) on 96-well plates. At least five clones were randomly selected and subjected to tumorigenicity assay.

Treatments during anchorage blockade

For anchorage blockade assays, adherent melan-a melanocytes were harvested by mild trypsin treatment and 1×10^5 cells/ml were cultured on 1% (w/v) agarose-coated plates for 3 h (D3h) under the same conditions described above. Alternatively, melan-a cells were treated with 4 mM 2,4-diamino-6-hydroxypyrimidine (DAHP), 40 μM L-sepiapterin, 1 mM L-NAME, or 4 μM ebselen (all from Cayman Chemical) in 0.5% (v/v) FBS-supplemented RPMI for 16 h, or with 100 μM apocynin (Calbiochem), 50 μM Mn(III) tetrakis (4-benzoic acid) porphyrin (Mn(III)TBAP; Cayman Chemical), and 500 μM 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1*H*-imidazolyl-1-oxy-3-oxide (PTIO; Calbiochem) for 2 h in 0.5% (v/v) FBS-supplemented RPMI, or with 1 μM antimycin (Sigma, Steinheim, Germany) and 5 μM rotenone (Sigma) for 3 h in 0.5% FBS-supplemented RPMI. After treatment, the cells were harvested and placed on agarose-coated plates (1×10^5 cells/ml) for 3 h (D3h) with or without the inhibitors or scavengers described above. In parallel, adherent melan-a melanocytes were maintained for an additional 3 h in the presence of inhibitors or scavengers before being harvested (D0).

Superoxide measurement

Intracellular superoxide levels were measured using dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA), a nonfluorescent cell-permeative indicator for superoxide, as previously described [20]. Adherent and suspended melan-a cells and melan-a-derived cell lines were washed and incubated in PBS for 30 min at 37 °C before being incubated with 200 ng/ml DHE for an additional 30 min at 37 °C in the dark. After being washed, the cells were analyzed by flow cytometry (FACSCalibur; Becton–Dickinson, Franklin Lakes, NJ, USA) (excitation wavelength 480 nm; emission wavelength 567 nm). Alternatively, superoxide generation was determined by chemiluminescence using coelenterazine (Calbiochem) as previously described [21]. Briefly, 1×10^5 cells were seeded in 96-well plates (D0) or subjected to adhesion impediment for 3 h (D3h). After this period, 2 μM coelenterazine was added to the medium and chemiluminescence was assessed immediately in a luminometer (Softmax Pro; Molecular Devices, Sunnyvale, CA, USA).

Nitric oxide measurement

Extracellular NO levels in adherent (D0) and de-adherent melan-a cells (D3h), treated or not with the scavengers described above, and in melan-a-derived cell lines were determined after a gas-phase chemiluminescence reaction of NO with ozone by a NO analyzer (NOA 280; Sievers Instruments, Boulder, CO, USA). A standard curve was established with a set of serial dilutions (0.1–100 μM) of sodium nitrate. The concentrations of NO metabolites in samples were determined by comparison with a standard curve and expressed as micromoles per milligram of protein. Data collection and analysis were performed using the NOANalysis software (version 3.21; Sievers Instruments). Alternatively, the intracellular NO levels were measured using the fluorescence NO indicator diaminofluorescein-2-diacetate (DAF-2DA; Molecular Probes) in adherent cells (D0), suspended cells (D3h), and melan-a-derived cell lines. Cells were incubated with 10 μM DAF-2DA at room temperature for 30 min, rinsed with PBS, and analyzed by flow cytometry in a FACScan (Becton–Dickinson) (excitation wavelength 495 nm; emission wavelength 515 nm).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability in the presence of various concentrations of inhibitors and scavengers was estimated using a standard MTT assay (Sigma). Melan-a cells (2×10^4 cells/ml) were cultured in complete medium for 24 h on 96-well plates. The medium on the cell cultures was replaced by fresh medium containing 0.5% (v/v) FBS and then the cells were treated for 16 h with L-sepiapterin (10, 20, 40, 60, 80, and 100 μM), ebselen (1, 2, and 4 μM), or DAHP (1, 2, 4, 8, 10, and 12 mM), or for 2 h with PTIO (125, 250, and 500 μM), Mn(III)TBAP (25, 50, and 100 μM), or apocynin (25, 50, 100, 200, and 400 μM), or for 3 h with rotenone (5, 10, and 20 μM) or antimycin (0.25, 0.5, and 1 μM) at 37 °C in 5% CO_2. The absorbance at 570 nm was recorded in each well using an ELISA microplate reader. Melan-a and Tm5 cell viability under anchorage blockade conditions was also estimated by MTT assay. For anchorage blockade assays, adherent melan-a and Tm5 cells were harvested by mild trypsin treatment and 2.5×10^3 cells were cultured per milliliter on 1% (w/v) agarose-coated plates for 96 h (D96h) in the absence or presence of 50 μM Mn(III)TBAP or 20 μM L-sepiapterin in

fresh medium containing 5% (v/v) FBS. Viability of adherent melan-a and Tm5 cells after treatment for 96 h under the same conditions as described above was also evaluated.

Trypan blue dye exclusion assay

Cell viability of adherent melan-a and Tm5 cells was evaluated using the trypan blue dye exclusion assay. Melan-a and Tm5 cells (2×10^4 cells) were cultured in complete medium on six-well plates. After 24 h the medium was replaced by fresh medium containing 0.5% (v/v) FBS and the cells were treated for 16 h with 20 μM L-sepiapterin. or the medium was replaced by fresh medium without FBS for 2 h with 50 μM Mn(III)TBAP. Subsequently, both cell lines were treated for 96 h in complete medium with 20 μM L-sepiapterin or 50 μM Mn (III)TBAP. Adherent melan-a and Tm5 cells were harvested by mild trypsin treatment and suspended in 1 ml of complete medium. Trypan blue 0.4% solution (Molecular Probes) was added to an equal volume of cell suspension and the cells were counted in a Neubauer chamber.

Clonogenic cell-survival assay

Melan-a and Tm5 cells were cultured in complete medium for 24 h on six-well plates. The medium on the cell cultures was replaced by fresh medium containing 0.5% (v/v) FBS and then the cells were treated for 16 h with 20 μM L-sepiapterin or for 2 h with 50 μM Mn (III)TBAP. Adherent melan-a and Tm5 cells were harvested by mild trypsin treatment and 2.5×10^3 cells/ml were cultured on 1% (w/v) agarose-coated plates for 96 h (D96h) in the absence or presence of 50 μM Mn(III)TBAP or 20 μM L-sepiapterin in fresh medium containing 5% (v/v) FBS at 37 °C in 5% CO_2. Melan-a and Tm5 cells were collected by centrifugation, seeded on 60-mm dishes and grown for 3 days, for Tm5 cells, or 5 days, for melan-a cells, to allow colony formation. Colonies were washed with PBS, fixed in 3.7% (v/v) formaldehyde for 15 min, stained with 1% toluidine blue for 5 min, and washed with water. For quantification of surviving cells, the staining was dissolved in 1% SDS and the absorbance at 570 nm was evaluated using an ELISA microplate reader.

In vitro cytotoxic effects of carboplatin

Tm5 cells (2×10^5) were seeded in 96-well plates in culture medium supplemented with 5% (v/v) FBS. After being seeded, the Tm5 cells were incubated in the presence or absence of 50 μM Mn(III) TBAP in fresh medium containing 0.5% (v/v) FBS. After 16 h, the medium was replaced by fresh medium with 100 μM carboplatin in the presence or absence of 50 μM Mn(III)TBAP and the cells were incubated for an additional 48 h. The cytotoxic effect of carboplatin was evaluated using the MTT assay or trypan blue dye exclusion assay as described. The absorbance at 570 nm was recorded in each well using an ELISA microplate reader.

Tumorigenicity assays

The sequential anchorage blockade protocol resulting in melanocyte malignant transformation was performed by subjecting adherent melan-a cells pretreated with 1 mM L-NAME for 16 h to sequential cycles of substrate adhesion impediment in the presence of 1 mM L-NAME. Cells from subconfluent monolayers were harvested after trypsin treatment, counted, and then suspended in PBS**.** The clones derived from sequential cycles of anchorage blockade in the presence of L-NAME (2×10^6 cells) and a positive control (4C11+, melanoma cell line derived from repetitive cycles of melan-a anchorage blockade) were injected subcutaneously into the flanks of 6- to 8-week-old C57BL/6 syngeneic female mice. Animals were kept under 12-h daylight cycles, without food restriction, and checked daily for tumor development. The tumor volume (mm^3) was measured using the formula $d^2\times D/2$, where d represents the minimum diameter and D the maximum diameter. Each experimental group consisted of at least four animals.

Statistical analysis

Unpaired nonparametric Student's t test was used to analyze differences between the means using GraphPad Prism version 4 for Windows (GraphPad, San Diego, CA, USA). The significance level was established at $p<0.05$.

Results

Melanocytes produce increased levels of superoxide and NO when maintained in suspension

Previous data from our lab showed that the sustained stress conditions characterized by augmented generation of NO, superoxide, H_2O_2, and peroxynitrite may have an important impact on epigenetic alterations observed along melanocyte malignant transformation associated with anchorage blockade [18]. Although adherent melanocytes produce detectable levels of both NO and superoxide, a significant increase in both of them is observed when these melanocytes are subjected to anchorage impediment for 3 h (Supplemental Fig. S1). The specificity of superoxide measurement by chemiluminescence using coelenterazine (Supplemental Figs. S1A and B) and by DHE staining (Supplemental Fig. S1C) and NO measurement by NO analyzer (Supplemental Fig. S1D) was confirmed by treating cells with the SOD mimetic Mn(III)TBAP and the NO scavenger PTIO.

eNOS is an important source of superoxide produced during melanocyte anchorage blockade

In all experiments the adherent cells were treated with various noncytotoxic concentrations of scavengers or inhibitors. To determine these concentrations, MTT assays were performed with the scavengers PTIO and Mn(III)TBAP (Supplemental Figs. S2A and B, respectively) and inhibitors (Supplemental Fig. S2C-I). Our previous data showing that treatment with the NOS inhibitor L-NAME impaired both the increase in DNMT protein levels and the global DNA hypermethylation observed during melanocyte anchorage blockade apparently indicated that NO had an important role in these epigenetic alterations [18]. However, superoxide, but not NO [18], production was inhibited in suspended melanocytes by L-NAME treatment. In fact, L-NAME itself can be a source of nonenzymatically produced NO [23,24]. Alternatively, L-NAME can impair the transfer of electrons to molecular oxygen, inhibiting superoxide production [11]. In this context, our results might be related to the involvement of NOS in the generation of superoxide by melanocytes maintained in suspension. Consistent with this, increased eNOS, but neither iNOS nor neuronal (n) NOS, expression was observed when melanocytes were maintained in suspension compared to adherent conditions ([18] and data not shown).

The fact that L-NAME addition resulted in a reduction (Figs. 1A and B), and not in the complete abolishment, of superoxide production suggests possible additional sources of superoxide in melanocyte anchorage impediment. To determine whether NADPH oxidases and mitochondria are those possible sources, melanocytes were treated with the NADPH oxidase inhibitor apocynin and the mitochondria inhibitors antimycin and rotenone. No statistically significant alteration was observed in superoxide production during melanocyte anchorage impediment in the presence of apocynin (Figs. 1C and D), antimycin, or rotenone (Figs. 1E and F).

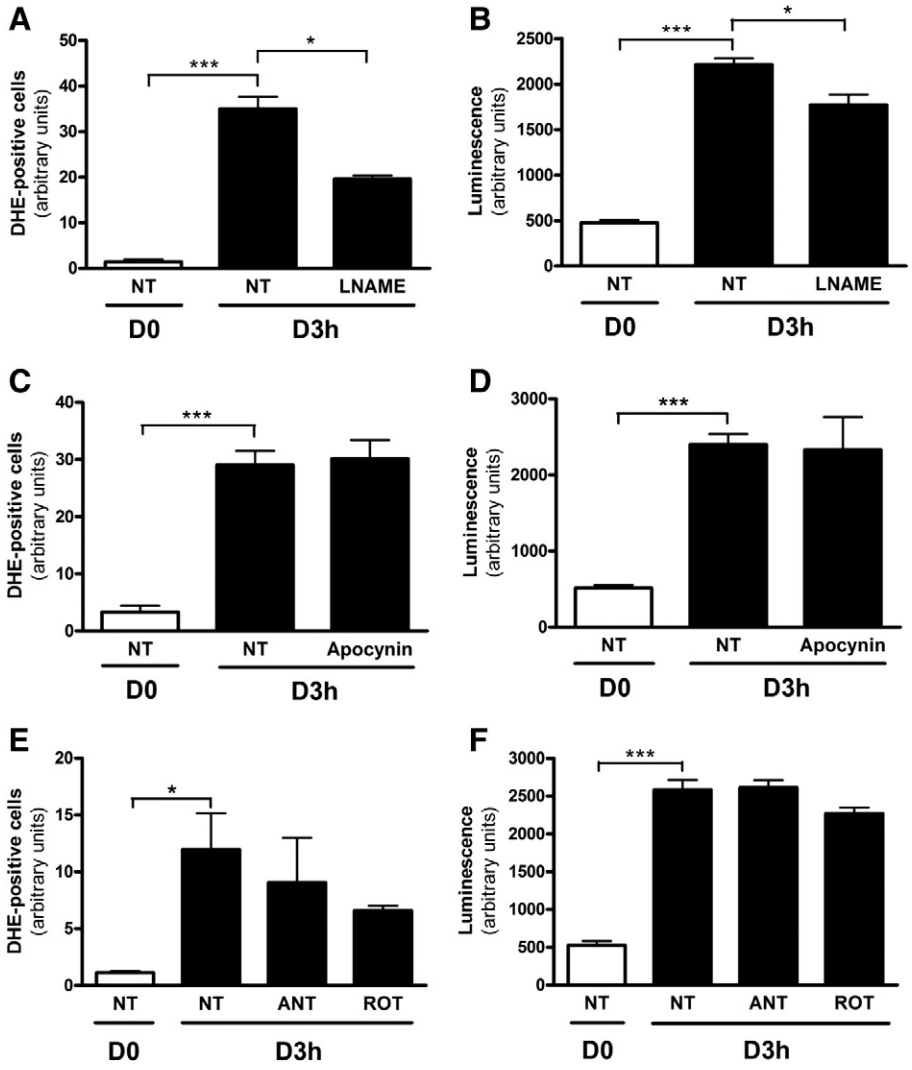

Fig. 1. eNOS is involved in superoxide production during melanocyte adhesion impediment. Melan-a cells were incubated or not (NT) with various inhibitors for 16 h; subjected or not (D0) to anchorage blockade for 3 h (D3h) in the presence of (A and B) 1 mM L-NAME, (C and D) 100 μM apocynin, or (E and F) 1 μM antimycin (ANT) and 10 μM rotenone (ROT); and analyzed for intracellular superoxide levels by flow cytometry, using dihydroethidium (DHE) (A, C, and E) or chemiluminescence using coelenterazine (B, D, and F). $^{*}p<0.05$, $^{***}p<0.0001$.

Evidence of eNOS uncoupling during melanocyte adhesion impediment

Several data have shown that abnormal regulation of eNOS can result in the production of superoxide when this enzyme becomes uncoupled [11,12]. One of the mechanisms underlying the uncoupled state of eNOS is a deficiency of the cofactor BH_4 [24]. Melan-a cells subjected to anchorage blockade in the presence of the BH_4 precursor L-sepiapterin presented decreased levels of superoxide (Figs. 2A and B). In addition, these cells presented augmented levels of superoxide after treatment with DAHP, a specific inhibitor of GTP cyclohydrolase I, the rate-limiting enzyme in BH_4 synthesis (Figs. 2C and D). These data strongly suggest that eNOS becomes uncoupled in suspended melan-a cells, leading to generation of increased levels of superoxide. BH_4 is susceptible to oxidation by peroxynitrite, forming dihydrobiopterin (BH_2) and ultimately biopterin [25]. The treatment with DAHP decreases the NO levels in melanocytes subjected to adhesion impediment (Fig. 2F), showing the role of BH_4 in maintaining NO levels. To analyze the possible involvement of peroxynitrite in eNOS uncoupling, melanocytes were treated with the peroxynitrite scavenger ebselen. The production of superoxide was reduced during anchorage blockade after melanocytes were treated with ebselen, suggesting that the presence of peroxynitrite could favor an eNOS uncoupling state (Fig. 2E).

Abrogating superoxide anion levels results in decreased melanocyte survival in suspension

The superoxide anion generated in the first hours of melan-a cell de-adhesion is maintained for at least 96 h in suspension (data not shown). The sustained oxidative stress seems to participate in melan-a cell survival in suspension as determined by MTT (Figs. 3B and E) and clonogenicity assay (Fig. 3C). In the presence of superoxide scavenger, Mn(III)TBAP (Figs. 3B and C), or the BH_4 precursor L-sepiapterin (Fig. 3E), the survival of melan-a cells subjected to anchorage impediment for 96 h was decreased. It is important to note that in adherent melan-a cells neither Mn(III)TBAP nor L-sepiapterin was toxic (Figs. 3A and D).

Melanoma lineages derived from melanocytes subjected to sustained stress also produce elevated levels of superoxide due to eNOS uncoupling

The intracellular levels of superoxide in melan-a-derived cell lines representing melanoma progression were analyzed using DHE staining. The result shows that both premalignant melanocytes (4C lineage) and melanoma lineages (4C11−, 4C11+, and Tm5 lineages) generate elevated levels of superoxide (Fig. 4A) and decreased levels of NO (Figs. 4B and C) compared to the parental lineage, melan-a. To

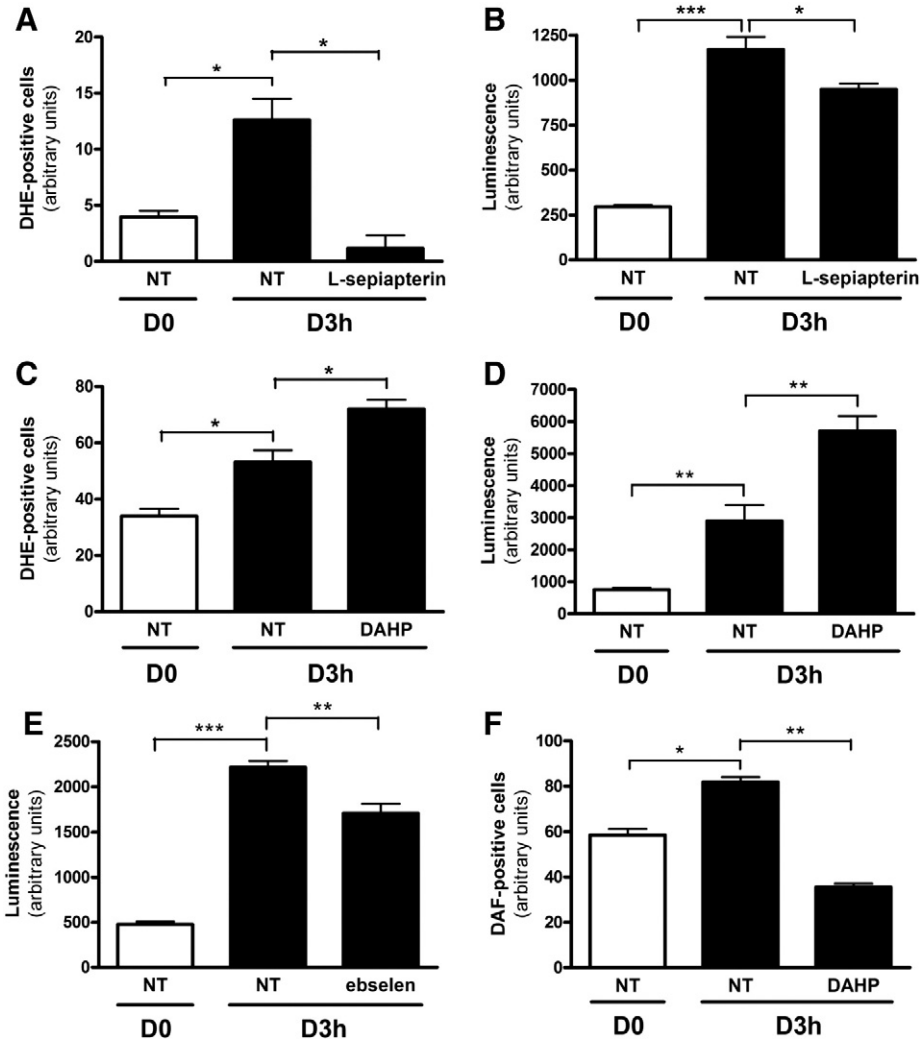

Fig. 2. Evidence of eNOS uncoupling in melanocytes subjected to anchorage blockade. Melan-a melanocytes were treated or not (NT) for 16 h with (A and B) 40 μM L-sepiapterin, a precursor of the eNOS cofactor tetrahydrobiopterin (BH_4), or (C, D, and F) 4 mM DAHP, a specific inhibitor of GTP cyclohydrolase I, the rate-limiting enzyme in the synthesis of BH_4, or (E) 4 mM ebselen, a peroxynitrite scavenger, and subjected or not (D0) to anchorage blockade in the presence of inhibitors for 3 h (D3h) and analyzed by flow cytometry for intracellular superoxide production, using DHE (A and C) or by chemiluminescence using coelenterazine (B, D, and E). (F) The NO production was evaluated by flow cytometry using DAF in melanocytes treated for 16 h with 4 mM DAHP and subjected to anchorage impediment for 3 h (D3h). *$p<0.05$, **$p<0.005$, ***$p<0.0001$.

investigate whether the increase in superoxide generation and reduction in NO levels in melanoma lineages was due to eNOS uncoupling, Tm5 melanoma cells were treated with the BH_4 precursor L-sepiapterin. The data show significant reduction in superoxide generation (Fig. 4D) and increase in NO levels (Fig. 4E) by melanoma cells in the presence of L-sepiapterin. Taken together, these results indicate that eNOS uncoupling is maintained along melanocyte malignant transformation, resulting in elevated levels of superoxide throughout neoplastic progression.

Abrogating superoxide levels results in decreased melanoma cell survival in suspension

Sustained oxidative stress seems to be important to the survival of suspended Tm5 melanoma cells as determined by MTT (Figs. 5B and E) and clonogenicity assays (Figs. 5C and F). We evaluated anoikis resistance in Tm5 melanoma cells treated or not with either the superoxide scavenger Mn(III)TBAP (Figs. 5B and C) or L-sepiapterin (Figs. 5E and F). Decreased superoxide levels rendered Tm5 melanoma cells more susceptible to anoikis when these cells were maintained in suspension for 96 h. Is important to note that neither Mn(III)TBAP (Fig. 5A) nor L-sepiapterin (Fig. 5D) was toxic to adherent Tm5 melanoma cells.

Decreased superoxide levels render Tm5 melanoma cells more sensitive to chemotherapy

One of the main characteristics of melanoma cells is their intrinsic chemo- and radioresistance. As we observed that superoxide has an important role in anoikis resistance, we next evaluated if increased superoxide levels also contribute to the drug-resistant phenotype of melanoma cells. Fig. 6 shows that treatment with superoxide scavenger, Mn(III)TBAP, overcomes this resistance mechanism and renders Tm5 melanoma cells more sensitive to cell death induced by carboplatin treatment as shown by both MTT (Fig. 6A) and trypan blue dye exclusion assays (Fig. 6B).

eNOS inhibition during de-adhesion cycles impairs melanocyte malignant transformation

To establish the importance of superoxide produced by eNOS uncoupling in melanocyte malignant transformation, the repeated

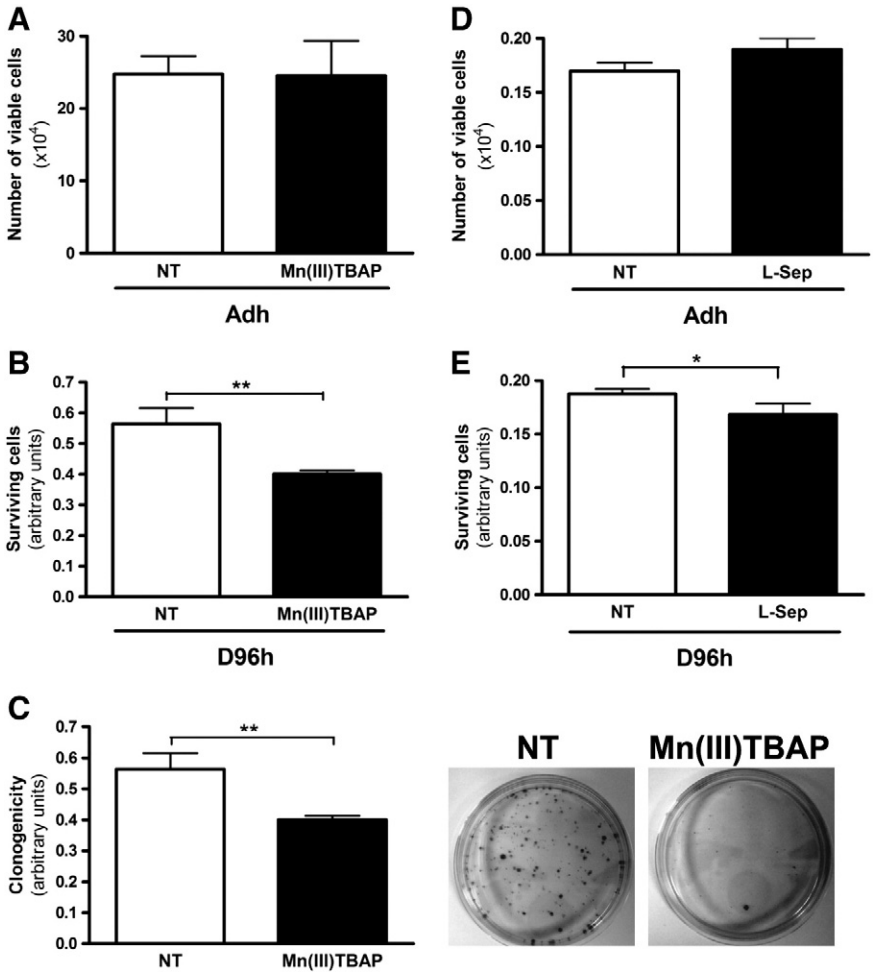

Fig. 3. Inhibition of superoxide levels renders melanocytes less resistant to survival in suspension. Adherent melan-a melanocytes were cultured in the presence of (A) 50 μM Mn(III) TBAP or (D) 20 μM L-sepiapterin for 96 h and viable cells were evaluated using trypan blue dye exclusion assay. Melan-a melanocytes were treated or not (NT) for (B and C) 2 h with 50 μM Mn(III)TBAP or (E) 16 h with 20 μM L-sepiapterin and subjected to anchorage blockade in the presence of inhibitors for 96 h (D96h), and surviving cells were estimated by MTT (B and E) or by clonogenicity assay (C). $^{*}p<0.05$, $^{**}p<0.005$.

cycles of anchorage blockade were performed in the presence of the NOS inhibitor L-NAME. As observed in Fig. 7, the treatment of melan-a cells with L-NAME before and during each de-adhesion cycle impaired the acquisition of a malignant phenotype as evaluated by the ability of cells to form tumors in vivo, reinforcing the fundamental role of eNOS uncoupling in this process.

Discussion

Our group has recently established a melanocyte malignant transformation model in which several melanoma lineages were obtained after subjecting immortalized, but nontumorigenic, melanocytes lineage (melan-a) to serial cycles of anchorage blockade followed by reattachment under normal culture conditions [17]. We have further demonstrated increased nitric oxide, superoxide anion, and hydrogen peroxide intracellular levels in the first hours of melanocyte adhesion impediment [18]. In addition, strong evidence was found for the involvement of superoxide anion, but not H_2O_2, generation in the epigenetic alterations observed during melanocyte anchorage blockade, because L-NAME, a NOS inhibitor, and *N*-acetylcysteine (NAC), an antioxidant, but not catalase or peroxidase, H_2O_2 inhibitors, abrogated the increase both in DNMT1 and DNMT3b expression and in global DNA methylation levels observed under this condition [18]. In addition to acting like an antioxidant, NAC increases the expression of MnSOD, decreasing the superoxide anion levels in lung tissue [26]. However, we cannot exclude the participation of other reactive oxygen species in the melanocyte malignant transformation and melanoma progression in our model. It is important to note that L-NAME treatment leads to decreased superoxide but not NO levels during de-adhesion. This phenomenon is in agreement with the literature, considering that the inability of L-NAME to inhibit NO synthesis in melanoma cells and in other cell types has already been demonstrated [22,23]. Nevertheless, L-NAME can impair the transfer of electrons to oxygen, inhibiting the superoxide anion generation by NOS [11]. Although in vitro biochemical studies demonstrated that NOS can independently produce superoxide by an enzymatic uncoupled state under certain pathological conditions [13], NOS uncoupling in cancer has not been described yet. The treatment of melan-a melanocytes with the superoxide scavenger Mn(III)TBAP and nitric oxide scavenger PTIO confirmed superoxide (Supplemental Figs. S1A, B, and C) and NO production (Supplemental Fig. S1D) during melan-a anchorage blockade.

The involvement of NADPH oxidases in malignant transformation and tumor progression has been reported by several prior studies [27,28]. Oncogenic Ras-transfected NIH-3 T3 fibroblasts have enhanced superoxide anion production by Nox1 and these ROS levels are functionally required for Ras transformation [29]. It was further demonstrated that Nox1-generated ROS mediate oncogenic Ras-induced up-regulation of VEGF and angiogenesis [30]. Previous work demonstrated the up-regulation of Nox4 expression in melanoma [31] and a recent study suggests the role of Nox4-generated ROS in melanocyte malignant transformation by regulating the growth of

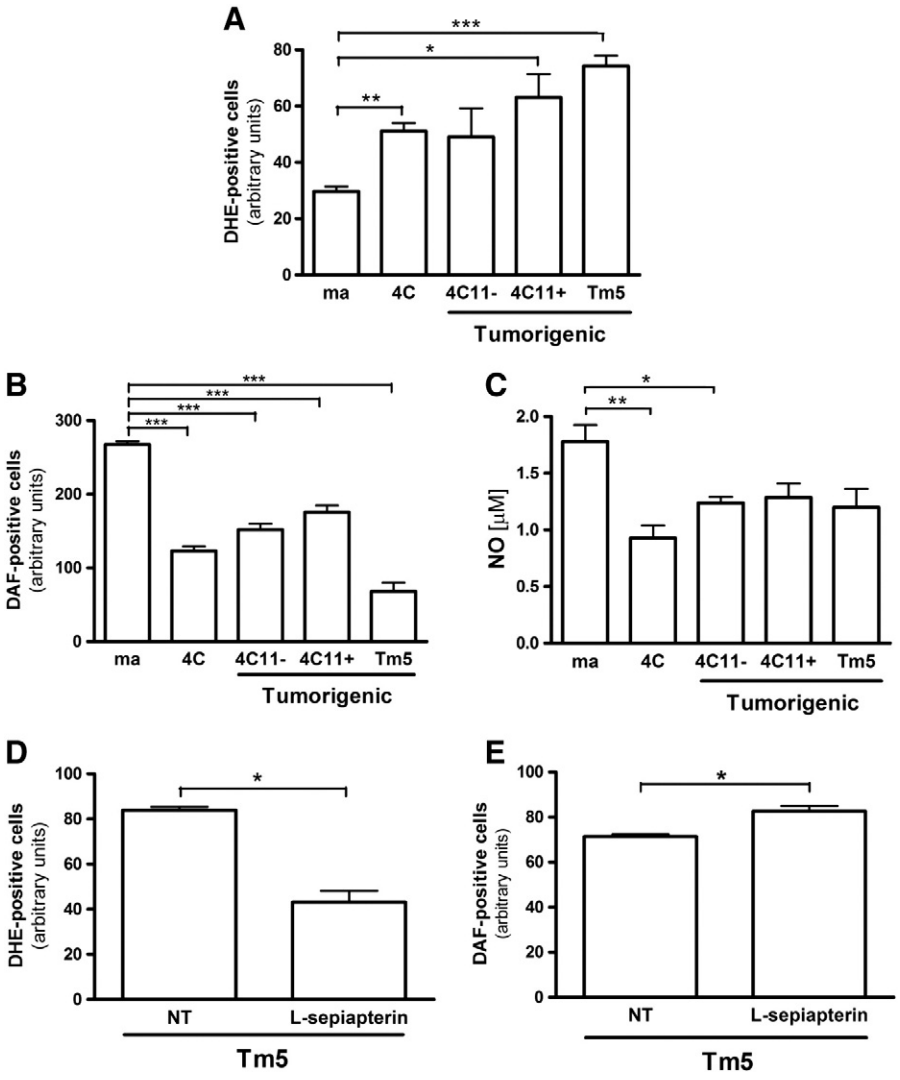

Fig. 4. The uncoupled eNOS seems to be maintained in melanoma cells derived from melan-a melanocytes subjected to sustained stress. (A) Intracellular superoxide levels in melan-a-derived cell lines were analyzed by flow cytometry using DHE and NO levels by (B) flow cytometry using DAF or by (C) NO analyzer. Tm5 melanoma cells were treated or not (NT) with 40 μM L-sepiapterin for 16 h and analyzed by flow cytometry for (D) intracellular superoxide levels using DHE or (E) intracellular NO levels using DAF. ma, melan-a melanocytes; 4 C, melan-a melanocytes subjected to four de-adhesion cycles; 4C11−, nonmetastatic melanoma lineage; 4C11+ and Tm5, metastatic melanoma lineages. $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0001$.

melanoma cells [32]. In our study, apocynin caused no changes in superoxide levels during melan-a melanocyte anchorage blockade (Figs. 1C and D). Apocynin was first reported to inhibit ROS production by activated neutrophils and has been used as an NADPH inhibitor in various cell types by many investigators [33,34]. However, to act like an NADPH oxidase inhibitor apocynin must be first metabolized by peroxidase to generate apocynin dimers, which then inhibit the translocation of the p47phox cytoplasmic subunit to the membrane assembly into active enzyme complex [35]. It has also been suggested that Nox4 activation does not require the translocation of p47phox to the membrane [36]. So, in fact, we cannot exclude NADPH oxidase as an additional source of superoxide in de-adhered melan-a melanocytes. The expression of NADPH oxidase isoforms in these cells is under investigation in our laboratory. Additional assays will be performed to analyze the NADPH oxidase activity in melan-a cells subjected to de-adhesion.

The main source of superoxide in the nonphagocytic cells is the mitochondrion. Although we have observed a loss of mitochondrial membrane potential during melanocyte de-adhesion (data not shown), the treatment with inhibitors of mitochondrial complexes I and III, respectively rotenone and antimycin, did not abrogate significantly the superoxide production by melan-a melanocytes subjected to anchorage blockade (Figs. 1E and F). These data indicate that the mitochondrion is not a source of superoxide anion production in melan-a cells maintained in suspension.

Uncoupled NOS can also be a source of superoxide under pathological conditions. Increasing evidence supports that uncoupled eNOS has a central role in endothelial dysfunction in vascular disease states [13]. Furthermore, impaired NO-mediated endothelial function with increased ROS generation is an independent risk factor for cardiovascular disease [37]. A critical aspect of NOS function is the BH_4 availability [24]. Maintenance and stabilization of NOS dimers are dependent on the presence of BH_4, because eNOS dimerization is essential for NO production. Other than that, BH_4 plays a crucial role in the multistep oxidation of arginine through the *N*-hydroxy-L-arginine intermediate and the subsequent generation of NO. Demonstration of eNOS uncoupling came from studies showing that BH_4 availability is essential to maintaining the electron transfer from NOS flavins coupled to L-arginine oxidation leading to NO generation. In the presence of suboptimal concentrations of BH_4, eNOS catalytic activity becomes uncoupled, leading to superoxide production and decreased NO production. Superoxide release was inhibited by the presence of

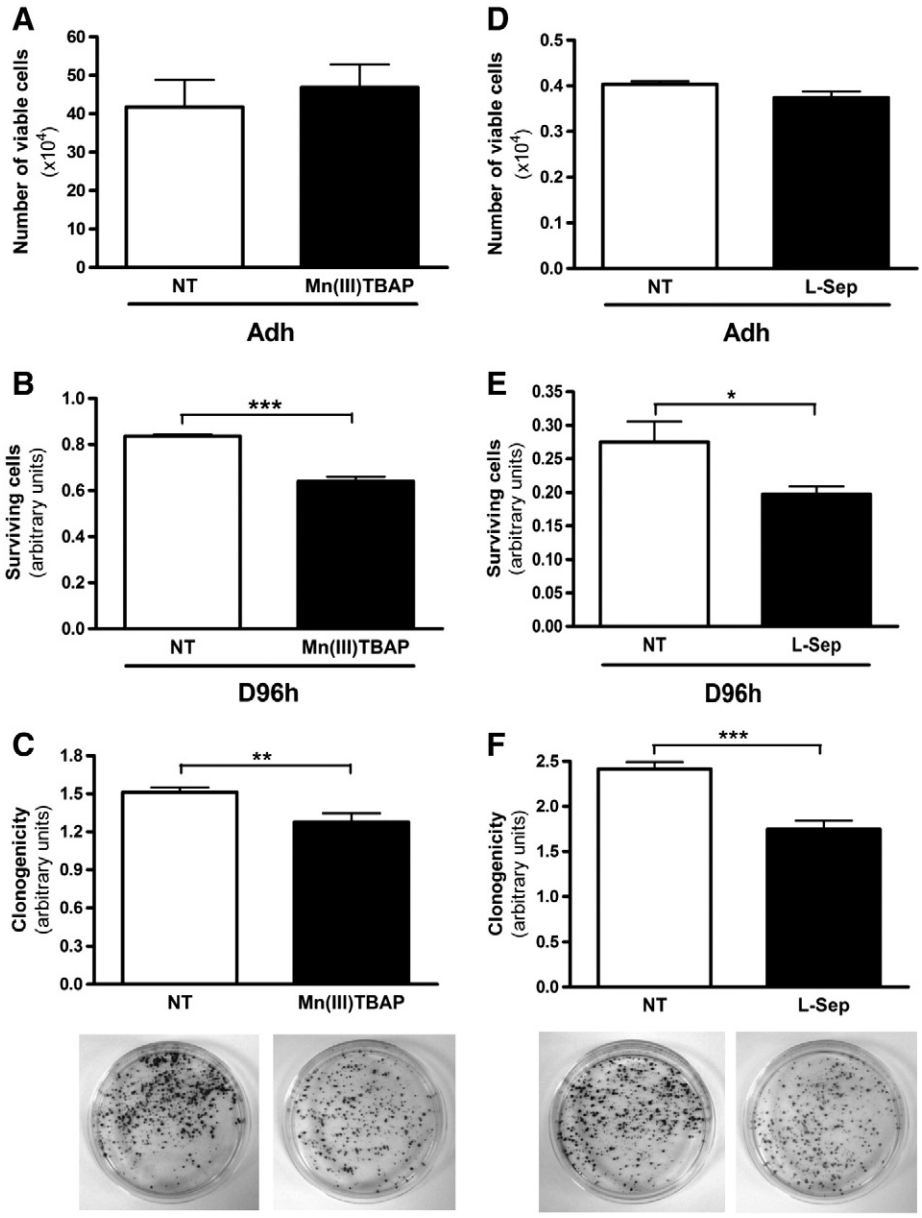

Fig. 5. Decreasing superoxide levels increase the susceptibility of melanoma cells to anoikis. Adherent Tm5 melanoma cells were treated or not for 96 h with (A) 50 μM Mn(III)TBAP or (B) 20 μM L-sepiapterin and viable cells were evaluated by trypan blue dye exclusion assay. Tm5 melanoma cells were subjected to anchorage blockade for 96 h (D96h) in the absence (NT) or presence of (B and C) 50 μM Mn(III)TBAP or (E and F) 20 μM L-sepiapterin and surviving cells were estimated by MTT (B and E) and clonogenicity assays (C and F). $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0001$.

BH_4 in various systems by pharmacological supplementation or genetic manipulations [38]. In agreement with this, treatment of melan-a cells subjected to anchorage blockade with L-sepiapterin, the BH_4 precursor, decreased superoxide anion levels (Figs. 2A and B). Various studies have revealed that pharmacological BH_4 supplementation improves endothelial function in those who smoke, diabetic or hypertensive subjects, patients with hypercholesterolemia, and those with coronary disease [39,40,41]. The cofactor BH_4 can be easily oxidized to form BH_2 and biopterin, both incapable of NO catalysis [37]. Peroxynitrite formed by NO/superoxide reaction could oxidize BH_4 as previously described and thereby promote eNOS uncoupling [42]. Our previous results have shown increased levels of malondialdehyde, one of the major aldehyde products of lipid peroxidation caused by peroxynitrite action, during melanocyte anchorage blockade [18]. In the presence of ebselen, a peroxynitrite scavenger, the superoxide level decreased significantly during melanocyte de-adhesion (Fig. 2E), suggesting that peroxynitrite stimulates superoxide production, probably by causing eNOS uncoupling by oxidizing BH_4 cofactor. In addition, it was recently shown that the striking linear relationship between eNOS protein and cellular BH_4 stoichiometry together with the intracellular BH_4:BH_2 ratio, rather than absolute concentrations of BH_4, is a key determinant of eNOS uncoupling, even in the absence of pathological disease [38,43]. Various kinds of stimuli can modulate NOS expression, including stress signals. Melan-a cells subjected to anchorage blockade present increased expression of eNOS [18], but not of nNOS or iNOS (data not shown). This augmentation of eNOS expression might contribute to disrupting the balance of the eNOS and BH_4 ratio, leading to partial eNOS uncoupling during de-adhesion, generation of superoxide, and a biochemical vicious cycle, in which BH_4 is oxidized, increasing eNOS uncoupling and consequently augmenting superoxide and reducing NO production in melan-a-derived cell lines. Our results showed that treatment of melan-a cells subjected to anchorage blockade with DAHP, an inhibitor of GTP cyclohydrolase I, the rate-limiting enzyme

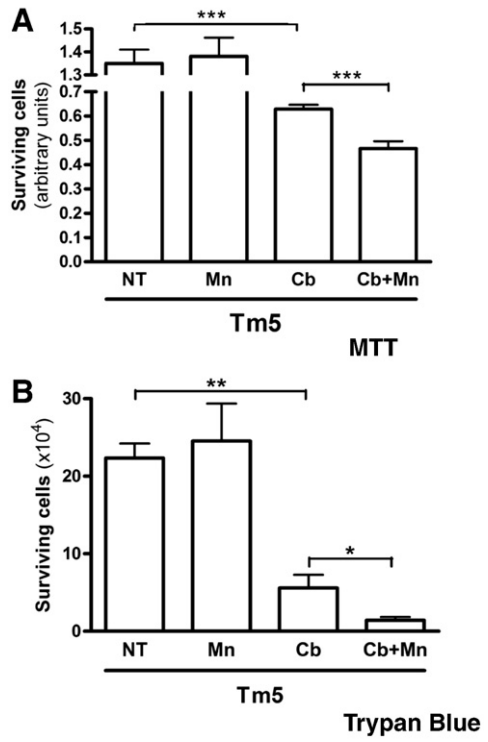

Fig. 6. Decreasing superoxide levels render Tm5 melanoma cells more sensitive to carboplatin chemotherapy. Tm5 cells were incubated in the presence (Mn) or absence (NT) of 50 μM Mn(III)TBAP in fresh medium without FBS. After 16 h, the medium was replaced by fresh complete medium with 100 μM carboplatin in the presence (Cb + Mn) or absence (Cb) of 50 μM Mn(III)TBAP. After 48 h, viable cells were estimated by (A) MTT and (B) trypan blue dye exclusion assays. $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0001$.

in BH_4 synthesis, leads to augmented superoxide (Figs. 2C and D) and decreased nitric oxide levels (Fig. 2F). Bendall and colleagues [43] showed that superoxide production increases in endothelial cells transfected with the eNOS transgene, but the overexpression of GTPCHI restored the basal superoxide levels to the wild-type cell levels, indicating the role of BH_4 in NOS coupling. Taken together, our data suggest the involvement of uncoupled eNOS in the generation of superoxide in melan-a cells subjected to anchorage blockade. It is important to note that eNOS uncoupling seems to be partial, because NO is also produced during melan-a melanocyte de-adhesion (Supplemental Fig. S1D).

Fig. 7. Decreasing superoxide levels impair melanocyte malignant transformation induced by sustained stress conditions. Melan-a melanocytes treated with L-NAME were subjected to sequential cycles of adhesion blockade. Cell clones (C3 LN, C4 LN, C5 LN, C6 LN) established after limiting dilution were subcutaneously injected into the flanks of syngeneic C57BL/6 female mice and tumor development was checked daily. One clone (4C11+ melanoma cells), obtained after subjecting melan-a melanocytes to sequential cycles of anchorage blockade in the absence of L-NAME treatment, was also injected into mice.

To our knowledge, at least, the only study suggesting the involvement of superoxide generation by uncoupled eNOS in cancer is that from Czesnikiewicz-Guzik and colleagues [34]. In that study, the authors showed that increased levels of superoxide in oral squamous cell carcinoma were inhibited by apocynin and L-NAME, identifying NADPH oxidase and NOS as sources of superoxide production [34]. On the other hand, the role of iNOS and NO generation in melanoma progression is well established. Previous report has shown increased expression of iNOS in melanoma and gain of antiapoptotic function after constitutive production of endogenous NO by iNOS, promoting survival of melanoma human cells [44,45].

One of the hallmarks of cell transformation is the acquisition of anchorage-independent growth and anoikis resistance [46]. Previous studies demonstrated the role of superoxide in apoptosis resistance. Inhibition of superoxide production by various inhibitors, superoxide scavengers, or MnSOD transfection can sensitize tumorigenic cells to radiation, glucocorticoids, and chemotherapy-induced apoptosis [47,48]. In addition, there is evidence that MnSOD is a tumor suppressor gene in many cell types, because its expression suppresses the malignant phenotype of human breast, pancreatic, prostate, and melanoma cancer cells [49,50]. The survival of melan-a cells subjected to adhesion impediment for 96 h was evaluated in the presence of the superoxide scavenger Mn(III)TBAP. We observed that the maintenance of oxidative stress is associated with anoikis resistance because fewer cells survive if superoxide is sequestered by Mn(III)TBAP (Figs. 3B and C). In addition, melan-a melanocyte survival under anchorage-independent conditions was slightly, but significantly ($p<0.05$), decreased in the presence of the BH_4 precursor L-sepiapterin (Fig. 3E), showing the role of superoxide from eNOS in melan-a cell anoikis resistance. We can speculate that the sustained condition of superoxide generation by uncoupled eNOS during repetitive cycles of melan-a melanocyte anchorage impediment may contribute to the acquisition of an anoikis-resistant phenotype. The superoxide anion inhibition did not decrease the viability of adherent melan-a cells (Figs. 3A and D), showing the role of superoxide in survival of cells subjected to stress conditions such as the absence of cell–matrix interactions. We have shown that cell–matrix interactions are fundamental for maintaining the melanocyte normal phenotype, because sequential de-adhesion cycles lead to neoplastic transformation [17].

The uncoupled eNOS seems to be maintained along melan-a melanocyte malignant transformation, as we observed high levels of superoxide (Fig. 4A) and decreased levels of NO (Figs. 4B and C) in premalignant (4C cell line) and melanoma cells (4C11−, 4C11+, and Tm5 cell lines). The treatment of Tm5 melanoma cells with BH_4 precursor, L-sepiapterin, diminished the superoxide levels (Fig. 4D) and augmented the NO levels (Fig. 4E), reinforcing the participation of eNOS in the elevated superoxide production and indicating one more time the uncoupled state of this enzyme along melanoma progression. The treatment with L-sepiapterin led to a decrease in superoxide anion level and a less important increase in NO level. This condition was also observed in other cellular systems [27,43] and may be explained by the general antioxidant properties of BH_4 other than its function as a NOS cofactor.

Tm5 melanoma cells are more resistant to anoikis than melan-a melanocytes [51] and abrogation of superoxide production also renders Tm5 cells more sensitive to anoikis. Treatment with Mn(III)TBAP (Figs. 5B and C) or L-sepiapterin (Figs. 5E and F) decreased Tm5 melanoma cell survival in de-adherent conditions as demonstrated by MTT (Figs. 5B and E) or clonogenicity assay (Figs. 5C and F). Adherent Tm5 melanoma cell viability did not decrease after Mn(III)TBAP (Fig. 5A) or L-sepiapterin treatment (Fig. 5D). To develop metastases, tumorigenic cells must detach from primary tumor and survive without cell and matrix interactions. Our results show the involvement of superoxide in one of the hallmarks of tumor progression, anoikis resistance. Tm5 melanoma cells were treated with the chemotherapeutic carboplatin in the presence or absence of superoxide anion scavenger, Mn(III)TBAP. Our data showed that carboplatin is more toxic to Tm5 melanoma cells in

the presence of superoxide as evaluated by MTT (Fig. 6A) and trypan blue dye exclusion assays (Fig. 6B). So, the maintenance of oxidative stress seems to be important not only in malignant transformation, but also for abilities acquired during tumor progression such as the metastatic process and drug resistance. Our result is in accordance with Pervaiz and colleagues [47], who showed that a pro-oxidant environment provides resistance to drug-induced cell death in melanoma cells. They demonstrated that regulation of tumor cell sensitivity to apoptotic signals by superoxide may involve the activation of caspase signaling pathway. The targets of superoxide anion in our model are under investigation, but we observed the involvement of the Ras–Erk pathway in superoxide generation (unpublished results).

Finally, we showed the importance of superoxide generation by uncoupled eNOS in melanocyte malignant transformation induced by sustained anchorage impediment. Melan-a melanocytes subjected to sequential cycles of anchorage blockade in the presence of L-NAME, an exogenous NOS inhibitor, do not acquire a malignant phenotype (Fig. 7). Because L-NAME treatment abrogated the superoxide generation during melanocyte anchorage blockade [25], we may suggest that superoxide has a key role in melanocyte malignant transformation. Melan-a cells subjected to anchorage blockade presented altered global DNA methylation levels [18]. The global DNA hypermethylation observed in these cells is inhibited by L-NAME, indicating the importance of superoxide in epigenetic alterations [18]. The malignant transformation is a result of growth deregulation due to genetic and epigenetic alterations. Our hypothesis is that the sustained stress caused by anchorage blockade leads to an epigenetic reprogramming, altering the expression of key genes involved in malignant transformation. Therefore, L-NAME could be abrogating epigenetic reprogramming that is triggered by increased superoxide levels and consequently the melanocyte malignant transformation. In accordance with this, data from our laboratory showed that increased DNMT expression and global DNA hypermethylation observed in melan-a melanocytes subjected to anchorage impediment were inhibited by the superoxide scavenger Mn(III)TBAP (unpublished results).

In conclusion, we have demonstrated in this study that superoxide generation by eNOS uncoupling contributes to melanocyte malignant transformation, mediated probably by the loss of BH_4 availability. These mechanisms are potential key targets for therapeutic interventions in melanoma disease. Further studies should consider the effectiveness of BH_4 treatment in melanoma.

Supplementary materials related to this article can be found online at doi: 10.1016/j.freeradbiomed.2011.02.022.

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