Free Radical Biology & Medicine 50 (2011) 1263–1273

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

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

Fabiana H.M. Melo [a], Fernanda Molognoni [a,b], Alice S. Morais [b], Mariana Toricelli [b], Margareth G. Mouro [c], Elisa M.S. Higa [c], José D. Lopes [a,1], Miriam G. Jasiulionis [a,b,*,1]

[a] Disciplina de Imunologia, Universidade Federal de São Paulo, 04023–900 São Paulo, SP, Brazil
[b] Disciplina de Farmacologia, Universidade Federal de São Paulo, 04023–900 São Paulo, SP, Brazil
[c] Disciplina de Nefrologia, Universidade Federal de São Paulo, 04023–900 São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 17 August 2010
Revised 16 February 2011
Accepted 22 February 2011
Available online 15 March 2011

Keywords:
eNOS uncoupling
Superoxide anion
Melanocytes
Anoikis
Malignant transformation
Free radicals

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.

* Corresponding author. Fax: +55 11 5572 3328.
E-mail address: mgjasiulionis@unifesp.br (M.G. Jasiulionis).

[1] These authors share senior authorship.

0891-5849/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2011.02.022

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