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Decreased Mitochondrial Nitric Oxide Synthase Activity and Hydrogen Peroxide Relate Persistent Tumoral Proliferation to Embryonic Behavior1

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ABSTRACT

Differential expression and activity of constitutive mitochondrial nitric oxide synthase (mtNOS) in the mitochondrial compartment is followed by significant variations in matrix nitric oxide (NO) steady-state concentration. The mitochondrial utilization of NO involves the production of superoxide anion and H2O2, a species freely diffusible outside the mitochondria that participates in the modulation of cell proliferation and apoptosis and in cell transformation and cancer. On these bases, we analyzed the modulation of mtNOS in the frame of cellular redox state in M3, MM3, and P07 murine tumors and their respective cell lines, as compared with normal proliferating and quiescent tissues. The results showed that: (a) tumoral and proliferating mitochondria only retain 10–50% of the activity of complexes I, II–III, and IV and Mn-SOD of quiescent tissues; (b) normal proliferating tissues, like embryonic liver or pregnant mammary gland, have 10–20% of mtNOS expression and activity and mitochondrial H2O2 yield than quiescent nonproliferating tissues; (c) similarly but irrespective of mtNOS expression, tumoral mitochondria have no >5% of mtNOS activity and H2O2 yield of mature tissues; and (d) in opposition to stable tissues, both tumoral and normal proliferating cells exhibit high cyclin D1 expression and low pro-apoptotic p38mitogen-activated protein kinase activity. Dually, H2O2 stimulated tumor cell proliferation (<10 μM) or markedly inhibited it (>10 μM) with parallel variations of cyclin D1, phospho-extracellular-regulated kinase 1/2, and phospho-p38mitogen-activated protein kinase. It is surmised that decreased oxidative phosphorylation, defective tumoral mtNOS, and low mitochondrial NO-dependent H2O2 may be a platform to link persistent tumoral growth to embryonic behavior.

INTRODUCTION

Cancer is a disease in which unremitting clonal expansion of somatic cells kills by invading, subverting, and eroding normal tissues (1). Several lines of evidence indicate that tumorigenesis is a multistep process and that these steps reflect alterations that drive the progressive transformation of normal cells into highly malignant derivatives. It has been proposed that a deregulation of proliferation, together with an acquired resistance toward apoptosis, is a hallmark of most, perhaps all, types of cancer (2, 3).

In the last years, the role of nitric oxide in tumor biology has gained significance. Some canonical NOSs like iNOS have been consistently shown to be a variant or spliced version of the neuronal isoform nNOS (12, 13). This enzyme is constitutively expressed, requires the presence of calcium ion for activity, and is subjected to modulation by drugs (14) and hormones (15) or during development (13). It is noteworthy that changes in expression and activity of constitutive mtNOS will be followed by significant variations of matrix NO steady-state levels in the relatively small and well-differentiated mitochondrial compartment (15, 16). Furthermore, the utilization of NO involves the production of superoxide anion (O2•−) and hydrogen peroxide (H2O2), a species freely diffusible outside the mitochondria (17–19). Like this, the regulation of mitochondrial pathways for NO production and utilization may have a significant participation in life processes. In the last years, cumulative evidence showed that the fate of H2O2 and consequent oxidative stress levels play an important role in the activation of signaling molecules, which control the complex machinery involved in cell proliferation, differentiation, apoptosis, and senescence (20). Moreover, redox state is clearly related to the activity of growth factors and cell transformation and cancer (21).

Considering that grading expression and activity of mtNOS modulates H2O2 and oxidative stress in normal tissues (13), the goal of the present work was to characterize mitochondrial activities and mtNOS in organelles from murine tumors and integrate the information in a frame of cellular redox state, mitochondrial NO steady-state concentration, and cell cycle progression.

MATERIALS AND METHODS

Experimental Animals. We used 8–12-week-old tumor-bearing BALB/c mice from the University Institute of Oncology Angel H. Roffo. Lung and liver from nontumor bearing BALB/c mice were used as controls. In some experiments, E19-P2 and adult P90 Wistar rats were used to obtain liver at representative developmental stages with respectively high proliferation rate or nonlonger proliferating; to same purposes, mitochondria of rat mammary gland just after parturition and P2-P90-isolated hepatocytes were appropriately used. Animals were maintained in accordance with ethics, current regulations, and standards of the NIH.

Tumors. Two BALB/c transplantable mammary adenoarcinomas (M3 and MM3) and a BALB/c transplantable lung adenoarcinoma (P07) were used. P07 and M3 appeared spontaneously in the lung and mammary gland of BALB/c mice, respectively (22, 23), whereas MM3 variant resulted from successive s.c. trocar implants of M3 lung metastases into the flank of syngeneic mice (24). The three tumors, maintained by s.c. tumor trocar implants, are well characterized; M3 tumor has 40% incidence of lung metastasis, whereas MM3 variant which shows a longer tumor latency period than M3 (11 ± 2 versus 6 ± 2 days), develops lung metastases in 95% of the inoculated mice. P07 develops lung metastases in 100% of cases (22–24).

Cell Lines and Culture Conditions. LM3, LMM3, and LP07 cell lines were obtained from the respective tumors (25, 26). Comparatively, human breast adenocarcinoma MCF7 and normal murine mammary gland NMuMG cell lines from American Type Culture Collection were tested. LM3, LMM3, and LP07 cells were maintained in MEM (41500; Life Technologies, Inc.)

1 The abbreviations used are: mtNOS, mitochondrial nitric oxide synthase; NO, nitric oxide; DHCA-DTA, 2, 7-di-chlorofluorescin diacetate; Mn-SOD, manganese-superoxide dismutase; NOS, nitric oxide synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS; ATZ, 3-amino-1, 2, 4-triazole; L-NMMA, Nω-monomethyl-l-arginine; ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase.
cell lines derived from the studied tumors and human MCF7 mammary tumoral cell line. 1 cm² of mammary gland. between mtNOS activity and protein expression is represented in the different conditions. In B, carcinomas (D) were obtained after the purification assay. 

Table 1 Mitochondrial respiratory activities of normal and tumoral tissues

| (nmol c/min/mg prot) |
|---------------------|----------------|
| Normal adult quiescent tissues | 550 ± 46 |
| Mouse liver | 511 ± 37 |
| Mouse lung | 429 ± 46 |
| Normal proliferating tissues | 170 ± 28² |
| Rat E19 fetal liver | 225 ± 18³ |
| Rat pregnant mammary gland | 22 ± 3² |
| Tumors | 105 ± 15⁰ |
| MM3 | 103 ± 14⁰ |
| MM3 | 122 ± 6³ |

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<th>Complex I-III Complex II-III</th>
<th>Complex IV (kcat/min/mg prot)</th>
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<tr>
<td>Normal adult quiescent tissues</td>
<td>5 ± 1⁰</td>
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<td>Mouse liver</td>
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<td>Mouse lung</td>
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<td>Normal proliferating tissues</td>
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<td>Rat E19 fetal liver</td>
<td>22 ± 3²</td>
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<tr>
<td>Rat pregnant mammary gland</td>
<td>22 ± 3²</td>
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<td>Tumors</td>
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<td>P07</td>
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² Data are expressed as mean ± SE from 4–23 experiments by triplicate.
³ P < 0.05 vs. adult rat or mouse liver and lung.
⁰ Eight to 10 breasts/animal were pooled to obtain individual values for rat pregnant mammary gland.
⁵ P < 0.05 vs. tumors and E19 fetal liver.

Isolation and Purification of Mitochondria. Normal and tumoral tissues or cell lysates were washed and homogenized in MSHE (0.225 M mannitol, 0.07 M sucrose, 1 mM EGTA, and 25 mM HEPES/KOH, pH 7.4) and centrifuged at 600 × g for 10 min, and the supernatant was centrifuged at 10,000 × g for 10 min to obtain a mitochondrial pellet (15). To remove broken mitochondria, contaminating organelles, and adsorption artifacts, mitochondria were purified by centrifugation at 95,000 × g in MSHE supplemented with 0.1% BSA and Percoll buffer (30% Percoll in MSHE; Ref. 27). The fraction with a density of 1.052–1.075 g/ml was collected and washed with high ionic strength solutions. The purified normal or tumoral mitochondrial fractions had no >5–7% of the activities of cytosolic lactate dehydrogenase or peroxysomal catalase, as measured by standard assays (11). Protein content was assessed by Lowry method.

For transmission electron microscopy, purified mitochondria were fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer containing 50 mM Tris (pH 7.4) and centrifuged at 600 × g for 10 min, and the supernatant was centrifuged at 10,000 × g for 10 min to obtain a mitochondrial pellet (15). To remove broken mitochondria, contaminating organelles, and adsorption artifacts, mitochondria were purified by centrifugation at 95,000 × g in MSHE supplemented with 0.1% BSA and Percoll buffer (30% Percoll in MSHE; Ref. 27). The fraction with a density of 1.052–1.075 g/ml was collected and washed with high ionic strength solutions. The purified normal or tumoral mitochondrial fractions had no >5–7% of the activities of cytosolic lactate dehydrogenase or peroxysomal catalase, as measured by standard assays (11). Protein content was assessed by Lowry method.

NOS Activity. NOS activity was assessed by conversion of L-[3H]arginine to L-[3H]citrulline with minimal modifications (28). Samples were frozen and thawed once, and activities were measured in 50 mM phosphate buffer, sup-

supplemented with 5% heat-inactivated FCS, 2 mM 1-glutamine, and 80 μg/ml gentamicin, defined as complete medium, in plastic flasks (Corning) at 37°C in a humidified 5% CO₂ atmosphere. Passages were made by trypsinization of confluent monolayers (0.25% trypsin and 0.02% EDTA in Ca²⁺-Mg²⁺-free PBS, 80 mM Na₂HCO₃, 20 mM NaHCO₃, and 100 mM NaCl). MCF7 were maintained in DMEM nutrient mixture F-12 HAM (DMEM; D-2906, Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS and 50 μg/ml gentamicin. NMuMG were maintained in DMEM supplemented with 10% FCS, 50 μg/ml gentamicin, and 10 μg/ml insulin.

Cell Lysates. Cell lines were plated in Petri dishes, serum deprived for 24 h, and then washed in PBS and collected by scraping. Cells were lysed in buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.5% NP40, 1 mM MgCl₂, 1 mM phenylmethylsulphonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 25 mM sodium fluoride, and 1 mM sodium orthovanadate. Lysates were centrifuged at 13,000 × g for 30 min at 4°C, and the supernatant was used to study cell signaling cascades by immune blotting or NOS activity. Protein concentration was assessed by Lowry method.

Fig. 1. Microphotographs of isolated mitochondria from normal mice lung (A) and pregnant mammary gland (C) and from the murine lung P07 (B) and M3 mammary adenocarcinoma (D) were obtained after the purification assay. 1 cm = 0.33 μm (line inset).

Fig. 2. Mitochondrial NOS is expressed in tumors. In A (left), representative Western blot and mtNOS densitometries of murine mammary M3, MM3, and lung P07 tumors (black bars) at controlled protein load are shown; tumoral mtNOS is compared with that of adult liver (gray) and proliferating tissues like E19 fetal liver and pregnant rat mammary gland (white). Mitochondrial protein (100 μg/lane) was separated in 7.5% denaturing polyacrylamide gels, and mtNOS bands were detected using anti-iNOS antibodies. Densitometry of mtNOS bands is expressed as mean ± SE from five separate experiments, in arbitrary units. In A (right), the mtNOS activity of the respective groups is shown; in the inset, the ratio between mtNOS activity and protein expression is represented in the different conditions. In B, it is shown a representative Western blot of cytosolic and mitochondrial NOS of the cell lines derived from the studied tumors and human MCF7 mammary tumoral cell line. *, P < 0.05 respect to adult liver.
plemented with 50 mM l-valine, in the presence of 100 mM l-Arg (mitochondria) or 20 mM l-Arg (homogenates), 100 mM NADPH, 0.1 mM calmodulin, 0.3 mM CaCl₂, 1 mM flavin adenine dinucleotide, 1 mM flavin mononucleotide, and 10 μM tetrahydrobiopterin (BH₄; pH 7.4). Specific activity was determined after subtracting the remaining activity in the presence of 10-fold excess L-NMMA or 2 mM EGTA.

**Mitochondrial Production of Hydrogen Peroxide.** H₂O₂ production was continuously monitored in a Hitachi F-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan) with excitation and emission wavelengths at 315 and 425 nm, respectively (17). The assay medium consisted of 50 mM buffer phosphate, 50 mM l-valine (pH 7.4), supplemented with 12.5 units/ml horseradish peroxidase, 250 μM p-hydroxyphenylacetic acid, and 0.15 mg/ml of mitochondrial protein/ml, with 10 mM succinate as substrate. The assay was started with a 0.1 mM l-Arginine or pulses of 0.05–0.15 mM NO or, comparatively, with 2 mM antimycin (13, 29). To assess specific mtNOS-dependent H₂O₂ production rates, 1 mM L-NMMA was added to the mitochondrial preparations; fluorometric variations were specifically inhibited by 3 mM catalase. To uniform the maximal H₂O₂ production rate, mitochondrial preparations were supplemented with 1 mM SOD-mimetic Mn-(III) tetrakis(4-benzoic acid)porphyrin chloride.

**Mitochondrial Electron Transfer Activities.** Cytochrome oxidase activity was determined by recording the oxidation of 50 mM reduced cytochrome c at 550 nm in a Hitachi 3000 spectrophotometer; ε₅₅₀ = 21 mM⁻¹ cm⁻¹. The rate of the reaction was determined as the pseudo-first order reaction constant and expressed as k (min⁻¹) mg protein⁻¹. NADH-cytochrome c reductase and succinate-cytochrome c reductase activities were assayed by following the reduction of 30 μM cytochrome c in the presence of 1 mM KCN and, with 150 μM NADH or 8 mM succinate, as electron donors.

**Antioxidant Enzymes.** Mn-SOD activity was determined spectrophotometrically by measuring the inhibition of 10 μM cytochrome c by 3.5 mM/ml xanthine oxidase/50 mM xanthine at 550 nm in 50 mM potassium phosphate buffer with 0.1 mM EDTA at 25°C (pH 7.8; Ref. 30). Catalase activity was determined by monitoring 10 mM H₂O₂ decay at 240 nm in 50 mM phosphate buffer, 0.1% Triton X-100 (pH 6.8). The rate of the reaction was determined as the pseudo-first order reaction constant and expressed as k (min⁻¹) mg protein⁻¹ (31). Glutathione peroxidase activity was measured by monitoring the oxidation of 0.15 mM NADPH at 340 nm (ε₃₄₀ = 6.22 nm⁻¹ cm⁻¹) in 100 mM phosphate buffer, 1 mM EDTA (pH 7.7), supplemented with 5 mM reduced glutathione, 1 mM sodium azide, 0.25 unit of glutathione reductase, and 0.5 mM tert-butylhydroperoxide (32).

**Proliferation Assay.** Cells were plated in 96-well multiwell plastic dishes at the appropriate densities (3 × 10³ ML3 or LMM3 cells/well or 6 × 10³ LP07 in 0.2 ml complete medium and 6 × 10³ NMuMG cells/well in DMEM supplemented with 10% FCS and insulin) and allowed to attach overnight. Afterward, cells were supplemented with 0.1 mM to 1 mM H₂O₂ in MEM or DMEM without FCS. Comparatively, 5 mM catalase inhibitor ATZ was included 3 h before the treatment. At 48-h incubation, proliferation was assessed with a nonradioactive cell proliferation assay (Cell Titer 96; Aqueous Nonradioactive Cell Proliferation Assay; Promega, Madison, WI). The absorbance was measured with an ELISA plate reader at 492 nm.

**Flow Cytometry.** Tumoral and NMuMG cells were trypsinized and resuspended in HBSS with 1 mM CaCl₂ and 1 mM MgCl₂ (pH 7.4); neonate (P2) and adult (P90) hepatocytes were isolated as described previously (33). For flow cytometry, 10⁶ tumoral cells or hepatocytes were incubated in HBSS plus 5 μM...
DHCF-DA for 30 min at 37°C in darkness and washed once before determination; the assay was carried out in an Ortho Cytoron Absolute Flow-Cytometer (Johnson & Johnson). Propidium iodide (0.005%) was used to detect dead cells. For each analysis, 2 × 10^4 events were recorded.

**Western Blotting.** Western analysis using enhanced chemiluminescence detection system was carried out as described (13). The following primary antibodies were used: 1:500 dilution NOS and cyclin D1, 1:1000 dilution ERK1/2 and phospho-ERK1/2, and 1:250 p38 MAPK and phospho-p38 MAPK. Loading control was assessed by membrane staining with red ponceau.

**Reagents.** Cytochrome c, Cu/Zn SOD, xanthine, xanthine oxidase, calmodulin, tetrahydrobiopterin, Tris, NADPH, NADH, flavin adenine dinucleotide, flavin mononucleotide, sucrose, glucose, HEPES, EDTA, EGTA, succinate, glycerol, NP40, DTT, leupeptin, aprotinin, phenylmethylsulfonylfluoride, peptatin, Percoll, KCN, p-hydroxyphenylacetic acid, horseradish peroxidase, l-arginine, l-glutamine, L-NMMA, mannitol, BSA, antymycin A, Tween 20, Triton X-100, dichlorofluorescin diacetate (DHCF-DA), catalase, glutathione, hydrogen peroxide, and ATZ were from Sigma. SOD mimetic Mn-(III) tetrakis(4-benzoic acid)porphyrin chloride was from Calbiochem (San Diego, CA). Trypsin was from Life Technologies, Inc. (Grand Island, NY). Monoclonal antibody antineuronal NOS (N-31020) and polyclonal antiendothelial NOS (N-30300) were from Transduction Labs (Lexington, KY), polyclonal antimacrophage NOS (sc-650) and cyclin D1 antibody (sc-450) were from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-labeled antimouse antibody and antirabbit IgG horseradish peroxidase-linked antibody, acrylamide solutions, polyvinylidene difluoride membranes, and enhanced chemiluminescence kit were from Amersham Biosciences (Little Chalfont, United Kingdom). p38 MAPK antibody (#9212) and phospho-p38 MAP kinase antibody (#9211), and p44/42 MAPK antibody (#9102) and phospho p44/42 MAPK antibody (#9101) were from Cell Signaling Technology (Beverly, MA). l-[3H]Arginine was from NEN (Boston, MA). NO solutions (1.2–1.8 mM) were obtained by bubbling NO gas 99.9% purity (AGA GAS Inc., Maumee, OH) in water degassed with He by 30 min at room temperature and stored for a week at 4°C.

**Data Analysis.** One-way ANOVA and Sheffe post hoc comparisons were used to study the significance of the proliferation assay, activities of mitochondrial respiratory chain complexes, catalase and glutathione peroxidase activity, mtNOS activity, and intracellular concentration of reactive oxygen species as determined by flow cytometry.

**RESULTS**

**Mitochondrial Structure and Function.** After isolation and purification, normal and tumoral mitochondria were similar to the organelles observed in the original tissues. Tumor mitochondria were more bizarre and swollen than organelles obtained from the corresponding control tissues; variable shapes and sizes with loss of cristae and tendency to aggregate were also observed (Fig. 1).

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**Fig. 5.** The spontaneous H$_2$O$_2$ steady-state concentration of normal and tumoral cells. A, in the center, DCFH-fluorescence of LM3 (light gray), LMM3 (dark gray), and LP07 (black) murine tumor cells and from the immortalized NMuMG murine mammary gland cell line is compared with that of freshly isolated P2 proliferating neonatal hepatocytes (left) and P90 quiescent adult hepatocytes (right). Cells were incubated with 5 μM DHCF-DA by 30 min, washed twice, and examined in the flow cytometer. In all cases, fluorescence was determined in the live cell population as selected by 0.005% propidium iodide staining. B, peak fluorescence is plotted with cell NO-dependent [H$_2$O$_2$]$_{ss}$ estimated in accord to Eq. (1). Data are mean ± SE from three separate experiments; *, P < 0.05 versus adult hepatocytes; ***, P < 0.05 versus both adult and proliferating hepatocytes.
To test energy-linked functions, electron transfer rate was measured in the different populations with specific acceptors for segmental complex activities. Similarly to mitochondria from embryos or neonates, tumor organelles had low activities at complexes I-III, II-III, and IV, with respect to the adult liver ones (Table 1; \( P < 0.05 \)).

**mtNOS in Normal and Tumoral Tissues.** mtNOS was expressed in normal and tumoral mitochondria from rodents and also in the human MCF7 cell line. However, the comparative mtNOS protein level depended on the analyzed tumor. In M3 and MM3 tumors, mtNOS expression was lower than in adult liver and resulted similar to those of E19 proliferating liver and pregnant mammary gland; in contrast, mitochondria from P07 tumor had a robust expression of mtNOS similar to those from adult liver (Fig. 2A, left). Nevertheless and whatever the protein expression, mtNOS activity appeared to be markedly decreased in all tumoral mitochondria and at the same level of fetal liver or pregnant mammary gland (Fig. 2A, right). Therefore, mtNOS activity to protein ratio resulted markedly lower in the tumors than in normal tissues (Fig. 2A, inset). As described previously (11–13), \( M_r 140,000 \) mtNOS protein was recognized here in normal and tumoral tissues by anti-iNOS and anti-nNOS antibodies. In the tumoral cells, the presence of a \( M_r 140,000 \) NOS with same immune reactivity was detected in the cytosol as well (Fig. 2B); eNOS was not detected in the different subcellular fractions (data not shown). Interestingly, in controls, mtNOS activity was dependent on \( \text{Ca}^{2+} \) concentration, whereas \( \text{Ca}^{2+} \) did not appreciably modify the mtNOS activity of the tumoral tissues. In the absence of \( \text{Ca}^{2+} \), NO activity was not detectable in any of the studied mitochondrial populations.

**mtNOS-dependent Production of \( \text{H}_2\text{O}_2 \).** Mitochondria is the main source of superoxide anion (\( \text{O}_2^- \)) and of its Mn-SOD-catalyzed product of dismutation, \( \text{H}_2\text{O}_2 \) (34). \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) production rates are dramatically increased by antimycin-induced inhibition of mitochondrial electron transfer at complex III. In this study, tumoral mitochondria had a noticeable slower \( \text{H}_2\text{O}_2 \) production rate with antimycin than organelles from adult liver; similarly, this parameter was 50% lower in proliferating E19 liver than in the adult organ (Fig. 3).

In the physiological setting, NO inhibits cytochrome oxidase and \( b-c1 \) site at complex III and increases the ubisemiquinone radical, which provides electrons to \( \text{O}_2 \) and enhances \( \text{O}_2^- \) formation (17, 18). In accord, when mitochondria were solely supplemented with NO substrate \( \text{L-arginine} \), adult liver organelles were able to reach \( \sim90\% \) of the maximal \( \text{H}_2\text{O}_2 \) production rate, as acquired with antimycin. In contrast, neither tumors nor embryonic or proliferating tissues were able to reach that percentage in the presence of the substrate. This effect agrees with a low matrix NO concentration, as determined by low mtNOS activities in the proliferating and tumoral tissues.

**The Response of Tumoral Mitochondria to NO.** In addition, tumoral mitochondria used NO differently to normal organelles. Likewise, peak NO-dependent \( \text{H}_2\text{O}_2 \) production rate of M3 and MM3 mitochondria was \( \sim50\% \) of that of adult mice liver, whereas P07 tumor mitochondria had an even poorer response (17% of adult liver; Fig. 4). Moreover, peak mitochondrial \( \text{H}_2\text{O}_2 \) yield was achieved at 0.25–0.75 \( \mu\text{M} \) NO in M3, MM3, and P07 and at 2 \( \mu\text{M} \) NO in the adult mice liver. In the different conditions, ascendant and descendent slopes of \( \text{H}_2\text{O}_2 \) production rates indicated the preferential dismutation of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) or at higher matrix [NO] to the formation of peroxynitrite, in accord with the constant rates for the respective reactions [1] and [2]:

\[
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

\[
\text{O}_2^- + \text{NO} \rightarrow \text{ONOO}^-
\]

Likewise, the tumoral curves indicate a limited response to NO and a relatively fast transition from reaction [1] to reaction [2]. In addition, tumors and proliferative tissues had 30–60% Mn-SOD activity of that of quiescent adult liver (\( P < 0.05 \); Table 2).

**Tumoral Cells and \( \text{H}_2\text{O}_2 \) Steady-state Concentration.** \( \text{H}_2\text{O}_2 \) diffuses outside mitochondria and contributes to cytosol steady-state concentration ([\( \text{H}_2\text{O}_2 \]). Ref. 34): [\( \text{H}_2\text{O}_2 \]) also depends on catalyzing enzymes like catalase and glutathione peroxidase, which, in tumors, had no >5–40% of adult liver activities (Table 2). Therefore, NO-dependent-[\( \text{H}_2\text{O}_2 \]) may be calculated according to equation 1

\[
[\text{H}_2\text{O}_2] = \frac{k_1 [\text{NO}] + k_2 [\text{NO}^2]}{k_1 + k_2 [\text{NO}]}
\]

\[\frac{k_1 [\text{NO}] + k_2 [\text{NO}^2]}{k_1 + k_2 [\text{NO}]}
\]

Fig. 6. Dual effects of \( \text{H}_2\text{O}_2 \) on tumoral cell proliferation. The effects of increasing \( \text{H}_2\text{O}_2 \) alone (●) or plus 5 \( \mu\text{M} \) catalase inhibitor ATZ (○) were evaluated in tumoral LM3, LMM3, and LP07 cell lines and in NMuMG cells. Data are mean ± SE from three experiments by octuplicate. *\( P < 0.05 \) respect to the basal condition without \( \text{H}_2\text{O}_2 \); #, respect to cells treated with \( \text{H}_2\text{O}_2 \) alone.
peroxidase-driven reaction; Ref. 34). With catalase inhibitor ATZ, they became very sensitive to H$_2$O$_2$ and B$_2$ (Fig. 5).

LMM3, and LP07 was higher than that of P2 proliferating hepatocytes magnitude higher (10$^8$/H11002) that, at similar mitochondrial H$_2$O$_2$ yield, mean fluorescence of LM3, LMM3, and LP07 was higher than that of P2 proliferating hepatocytes (r$^2$ = 0.8; Fig. 5B). It is noteworthy that, at similar mitochondrial H$_2$O$_2$ yield, mean fluorescence of LM3, LMM3, and LP07 was higher than that of P2 proliferating hepatocytes (Fig. 5B; P < 0.05); likely, an intermediate tumoral cell fluorescence integrated the low H$_2$O$_2$ yield and very low catalase and glutathione peroxidase activities (Table 2; Eq.1).

H$_2$O$_2$ Modulates Tumoral Cell Proliferation. To relate redox status to cell cycle activity, we studied tumoral cell lines at different H$_2$O$_2$ concentrations. As shown in Fig. 6, tumoral cells displayed a biphasic response characterized by increased proliferation pattern at relatively low H$_2$O$_2$ concentration (0.1–10 $\mu$M, average 1 $\mu$M H$_2$O$_2$; 5–20%; P < 0.05) and a sharp inhibition of proliferation at higher H$_2$O$_2$ concentration; at 100–200 $\mu$M H$_2$O$_2$, cell cycle was completely arrested and/or death mechanisms were activated (P < 0.05). In contrast, normal NMuMG cell line with the lowest DHCF-[H$_2$O$_2$]$_m$ showed a prolonged proliferation phase, bearing concentrations as high as 500 $\mu$M H$_2$O$_2$. Accordingly to Eq (1), when cells were treated with catalase inhibitor ATZ, they became very sensitive to H$_2$O$_2$ and consequently, exhibited a marked decrease of proliferation respect to controls (Fig. 6; P < 0.001).

Cell Signaling in Tumoral Cell Lines and Proliferating Liver. We then studied the expression of cyclin D1, a protein involved in the control of cell proliferation as well as the expression and activity of ERK1/2 and p38MAPK, which are involved in proliferation, cell cycle arrest, and apoptotic pathways, and in the modulation of cyclin D1. Cyclin D1 was overexpressed in tumor cell lines, as well as in fetal rat liver (E19), whereas it displayed a low expression in quiescent rat adult liver, according to its nonproliferative state (Fig. 7). In contrast, p38MAPK had its highest expression in rat adult liver and was particularly active in this tissue (Fig. 7). In accord to the proliferation response to redox stress, cyclin D1 was dually modulated by H$_2$O$_2$ in the tumoral cells; in LP07 and at low 1 $\mu$M H$_2$O$_2$, cyclin D1 expression increased by 100%, whereas at high 50 $\mu$M H$_2$O$_2$, it decreased in ≤25% of controls. Depending on the length of their exposure time, a similar response to H$_2$O$_2$ was observed in LM3 cells (+25 and −75%, respectively; Fig. 8). At relatively prolonged exposure, H$_2$O$_2$ elicited differential responses in MAPKs activation; in LP07 and at 1 $\mu$M H$_2$O$_2$, phospho-ERK 1/2 increased by >15-fold and markedly declined at high H$_2$O$_2$ concentration, whereas phospho-p38 MAPK increased by 4-fold at 50 $\mu$M H$_2$O$_2$ (Fig. 8). No changes were observed in total MAPKs.

**DISCUSSION**

Recent studies propose common molecular and biological features between tumoral and embryonic tissues (35, 36). The results indicate that increasing mitochondrial NO and H$_2$O$_2$ yield is required in the transition from tissue proliferation to quiescence and that restriction of the specific mitochondrial pathways may contribute to enduringly deregulate tumor proliferation. In this context, this study analyzes for the first time a putative role of mitochondrial NO synthase in cancer.

The promotion of proliferation eventually entails a controlled inhibition of mitochondrial respiration (37). In support, most activities of tumoral and proliferating normal mitochondria were uniformly maintained at 20–30% of those of quiescent organelles (Table 1). Likewise, low electron transport-coupled ATP synthesis correlates with faster tumor growth (37) and high invasive behavior (38). In contrast, complete depolarization of mitochondrial membrane (39) or critical inhibition of complex I (40; also occurring at high matrix NO; Ref. 41) induce cell cycle arrest and apoptosis. In connection with down-regulated electron transfer, proliferating and tumoral mitochondria retained only 20–50% of the maximal H$_2$O$_2$ production rate of adult organelles (Fig. 3). According to Simmonet *et al.* (37), the differences between tumoral and embryonic mitochondria are particularly active in this tissue (Fig. 7). In accord to the proliferation response to redox stress, cyclin D1 was dually modulated by H$_2$O$_2$ in the tumoral cells; in LP07 and at low 1 $\mu$M H$_2$O$_2$, cyclin D1 expression increased by 100%, whereas at high 50 $\mu$M H$_2$O$_2$, it decreased in ≤25% of controls. Depending on the length of their exposure time, a similar response to H$_2$O$_2$ was observed in LM3 cells (+25 and −75%, respectively; Fig. 8). At relatively prolonged exposure, H$_2$O$_2$ elicited differential responses in MAPKs activation; in LP07 and at 1 $\mu$M H$_2$O$_2$, phospho-ERK 1/2 increased by >15-fold and markedly declined at high H$_2$O$_2$ concentration, whereas phospho-p38 MAPK increased by 4-fold at 50 $\mu$M H$_2$O$_2$ (Fig. 8). No changes were observed in total MAPKs.

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probably arise on the relative concentrations of mitochondrial respiratory components contributing to \( \text{O}_2^-/\text{H}_2\text{O}_2 \) formation, such as succinate-dehydrogenase (complex II) or ubiquinol (complex III). Interestingly, enzyme depletion and specific mutations in complex II genes lead to the development of tumors, like paraganglioma (42).

Recently, our group reported that mtNOS is subjected to developmental modulation (13). In this study, proliferating tissues linked up with low mtNOS activity and adult liver with high mtNOS activity; specific activity was preserved along development. In contrast, in the context of abnormal cells and mitochondria, markedly reduced specific activity and poor response to \( \text{Ca}^{2+} \) are congruent with the existence of a dysfunctional tumoral mtNOS.

Accordingly to NO utilization in mitochondria (18), modulation of mtNOS influences cell redox status and signaling (13, 29). Translational mtNOS increase renders a crescent H\(_2\)O\(_2\) yield, a pattern of H\(_2\)O\(_2\) formation, such as suc.

The “reciprocal dance between cancer and development” (35) is represented by similar cyclin D1 and MAPKs levels in tumors and developing/proliferating tissues. The expression of cyclin D1 and activation of proproliferative ERK1/2 or proapoptotic p38 MAPK in tumor cells were subjected to dual effects of H\(_2\)O\(_2\) (Fig. 8). It is surmised that: (a) redox modulation of tumor cell proliferation can take place through long-term sequential MAPKs activation (46); (b) in tumors, the balance of these signaling pathways results in uncontrolled growth; and (c) this effect encompass decreased mitochondrial activities and low H\(_2\)O\(_2\)\(_{\text{mm}}\). In addition, mitochondrial NO and H\(_2\)O\(_2\) influence other pro or antiapoptotic mitochondrial proteins, like bax (47).

Mitochondrial NOS could integrate a complex network with tumoral expression of other NOS isoforms; disruption of classic iNOS gene or NOS inhibition may, respectively, promote or suppress tumoral expression of other NOS isoforms; disruption of classic iNOS (47).

REFERENCES


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