Mitochondrial Nitric Oxide Synthase Drives Redox Signals for Proliferation and Quiescence in Rat Liver Development

María C. Carreras,1,2 Daniela P. Converso,1 Alicia S. Lorenti,3 Mariana Barbich,3 Damián M. Levisman,1 Ariel Jaitovich,1 Valeria G. Antico Arciuch,1 Soledad Galli,1 and Juan J. Poderoso1

Mitochondrial nitric oxide synthase (mtNOS) is a fine regulator of oxygen uptake and reactive oxygen species that eventually modulates the activity of regulatory proteins and cell cycle progression. From this perspective, we examined liver mtNOS modulation and mitochondrial redox changes in developing rats from embryonic days 17–19 and postnatal day 2 (proliferating hepatocyte phenotype) through postnatal days 15–90 (quiescent phenotype). mtNOS expression and activity were almost undetectable in fetal liver, and progressively increased after birth to tenfold up to adult stage. NO-dependent mitochondrial hydrogen peroxide (H2O2) production and Mn-superoxide dismutase followed the developmental modulation of mtNOS and contributed to parallel variations of cytosolic H2O2 concentration ([H2O2]ss) and cell fluorescence. mtNOS-dependent [H2O2]ss was a good predictor of extracellular signal–regulated kinase (ERK)/p38 activity ratio, cyclin D1, and tissue proliferation. At low 10–11–10–12 M [H2O2]ss, proliferating phenotypes had high cyclin D1 and phospho-ERK1/2 and low phospho-p38 mitogen-activated protein kinase, while at 10–9 M [H2O2]ss, quiescent phenotypes had the opposite pattern. Accordingly, leading postnatal day 2–isolated hepatocytes to embryo or adult redox conditions with H2O2 or NO-H2O2 scavengers, or with ERK inhibitor U0126, p38 inhibitor SB202190 or p38 activator anisomycin resulted in correlative changes of ERK/p38 activity ratio, cyclin D1 expression, and [3H]thymidine incorporation in the cells. Accordingly, p38 inhibitor SB202190 or N-acetyl-cysteine prevented H2O2 inhibitory effects on proliferation. In conclusion, the results suggest that a synchronized increase of mtNOS and derived H2O2 operate on hepatocyte signaling pathways to support the liver developmental transition from proliferation to quiescence. (HEPATOLOGY 2004;40:157–166.)

In normal adult liver, hepatocytes are highly differentiated and rarely undergo cell division, but they retain a remarkable ability to proliferate in response to acute or chronic injury.1 While liver regeneration depends on the transcriptional effects of cytokines, the mechanisms that govern developmental hepatocyte proliferation and the transition to the quiescent condition are not fully characterized.

At relatively low matrix steady-state concentration, NO exerts marked inhibitory effects on the activity of redox components of the electron transfer chain, particularly on cytochrome oxidase, thus regulating oxygen uptake.2,3 Consequently, the reduction level of mitochondrial components increases on the substrate side, leading to high superoxide anion (O2−) production; most of O2− is dismutated by matrix manganese superoxide dismutase (Mn-SOD) to hydrogen peroxide (H2O2) that freely diffuses outside the mitochondria.
Nitric oxide is vectorially released into matrix by mitochondrial nitric oxide synthases (mtNOSs). Different mtNOS isoforms have been described in rat tissues; liver mtNOS is a Ca\(^{2+}\)/calmodulin-dependent, constitutively expressed variant that is localized in the inner mitochondrial membrane. Elfering et al. reported 100% homology between liver mtNOS and neuronal nitric oxide synthase (NOS)-α by mass spectrometry; differentially, liver mtNOS has two posttranslational modifications: acylation with myristic acid and phosphorylation at C terminus, and a lower molecular weight (130 vs. 157 kDa). Additionally, mtNOS is subjected to selective C terminus, and a lower molecular weight (130 vs. 157 kDa). Additionally, mtNOS is subjected to selective modulation by thyroid status, cold acclimation, hypoxia, and brain plasticity.

Hydrogen peroxide and the consequent oxidative stress level play an important role in the activation of signaling molecules that control the complex machinery involved in cell proliferation, differentiation, apoptosis, and senescence. The major components of the cell cycle machinery are the cyclins and cyclin-dependent kinases. Cyclin D1 is implicated in the control of G1 phase progression in hepatocytes and other proliferating cell types, and its expression is positively regulated by the extracellular signal–regulated kinase (ERK) pathway and antagonized by stress-activated p38 mitogen-activated protein kinase (MAPK) cascade. During liver development, cyclin D1 content is inversely related to p38 MAPK activity, which in turn may be regulated by reactive oxygen species and NO.

Considering the mitochondrial utilization of NO and the NO-derived production of oxygen-active species, the regulation of mtNOS provides new insight into the physiological significance of mitochondria in cell biology. We report the modulation of mtNOS activity and the putative regulation of cell cycle redox signaling in the sequence of proliferating to quiescent cell stages during rat liver development.

**Materials and Methods**

**Animals.** Wistar rats were used in the experiments. National Research Council criteria for the care and use of laboratory animals in research were followed.

**Preparation of Whole Liver Homogenates.** Pooled liver of one litter from fetal (embryonic days 17 [E17] and 19 [E19]) and newborn rats at postnatal day 2 (P2), or whole liver of young and adult rats (P15–P90) were homogenized in 1 mL of cold lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 2.5 mM ethyleneglycol tetraacetic acid [EGTA], 1 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/mL each of aprotinin and leupeptin, 50 mM NaF and 0.1 mM sodium orthovanadate) per 100 mg of tissue. Tween 20 was then added at a final concentration of 0.1%. Homogenates were clarified by centrifugation at 10,000 g for 10 minutes at 4°C and stored at −70°C.

**Isolation and Purification of Liver Mitochondria.** Liver was homogenized in MSHE buffer (225 mM mannitol, 70 mM sucrose, 1 mM EGTA, 25 mM HEPES), pH 7.4 with 5 μg/mL each of aprotinin and leupeptin, 100 μg/mL PMSF, 10 μg/mL pepstatin, and 0.1% bovine serum albumin. The homogenate was centrifuged at 700 g at 4°C for 10 minutes; the supernatant was centrifuged at 7000 g for 10 minutes. A mitochondrial pellet was further purified using Percoll gradient to completely remove contaminating organelles and broken mitochondria. Purified mitochondria were tested for contamination by comparing lactate dehydrogenase activity (cytosolic marker) to succinate-cytochrome c reductase activity (mitochondrial marker); minimal contamination was found (2%–5%).

**Mitochondrial Enzyme Activities.** Nicotinamide adenine dinucleotide– and succinate-cytochrome c reductase activities (complexes I-III and II-III, respectively) were assayed by cytochrome c reduction at 550 nm with a Hitachi U3000 spectrophotometer (Hitachi, Tokyo, Japan) at 30°C. Cytochrome oxidase activity (complex IV) was determined by monitoring cytochrome c oxidation at 550 nm (ε\(_{550}\) = 21 mM\(^{-1}\) · cm\(^{-1}\)); the reaction rate was measured as the pseudo-first order reaction constant (k') and expressed as k'min\(^{-1}\) mg protein. \(^{20}\)

**NOS Activity.** NOS activity was determined through the conversion of [\(^3\)H]-L-arginine to [\(^3\)H]-L-citrulline in 50 mM of potassium phosphate buffer (pH 7.5) in the presence of 100 μM L-arginine, 0.1 μM [\(^3\)H]-L-arginine (NEN, Boston, MA), 0.1 mM NADPH, 0.3 mM CaCl\(_2\), 0.1 μM calmodulin, 10 μM tetrahydrobiopterin, 1 μM flavin adenine dinucleotide, 1 μM flavin mononucleotide, 50 mM L-valine and 1 mg/ml protein. Specific activity was calculated by subtracting the remaining activity in the presence of the NOS inhibitor N\(^{\text{t}}\)-methyl-L-arginine (5 mM L-NMMA) or 2 mM EGTA.

**Immunoblotting.** Cells were disrupted in lysis buffer (50 mM Tris buffer [pH 7.4]; 0.5% Nonidet P-40; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 10% glycerol; 1 mM MgCl\(_2\); 1 mM PMSF; 5 μg/mL each of aprotinin, leupeptin, and pepstatin; 1 mM sodium orthovanadate; 25 mM NaF; and 0.1 mM ammonium molybdate). Lysates were centrifuged at 12,000 g for 30 minutes at 4°C, and the supernatants were stored at −70°C. Western blot analysis was performed as described; membranes were stained with Ponceau red to ensure equivalent amounts of protein loading and electrophoretic transfer among sam-
pled. Western blotting enhanced chemiluminescence detection system and Hybond-P membranes were from Amersham Biosciences (Little Chalfont, United Kingdom). Quantification of bands was performed using digital image analysis (Total Lab, Nonlinear Dynamics, Newcastle, UK). Liver homogenates of lipopolysaccharide-treated rats, cerebellum cytosol, and endothelial cell lysates were used as inducible nitric oxide synthase (iNOS)-, neuronal nitric oxide synthase (nNOS)-, and endothelial nitric oxide synthase (eNOS)-positive controls. Proliferating liver from rats treated with a single dose of T3 (60 μg/100 g body weight, intraperitoneally) was used as a control for signaling assays.21

Co-immunoprecipitation. Mitochondrial or cytosolic proteins (500 μg) were incubated with 4 μg of polyclonal nNOS antibody and 30 μL protein A/G PLUS-Agarose (Santa Cruz, CA) at 4°C. The beads were then washed three times, suspended in sample buffer, boiled, and centrifuged, and the supernatants were subjected to immunoblotting against monoclonal nNOS antibodies.

Antibodies. Polyclonal anti-iNOS and anti-nNOS, anti-cyclin D2 and D3, and monoclonal anti–mouse cyclin D1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); polyclonal anti-eNOS and monoclonal nNOS antibodies were obtained from Transduction Laboratories (Lexington, KY). Polyclonal antibodies against total and phospho-p38 MAPK, ERK 1/2 and c-Jun N-terminal kinase, and phospho–MAPK kinases 1/2 were from Cell Signaling Technology (Beverly, MA).

Immunoelectron Microscopy. Purified mitochondria were suspended in 4% formaldehyde (pH 7.4) for 2 hours at 4°C, then dehydrated in 70%, 96%, and 100% ethanol (30 minutes for each step) and embedded in LR White. Immunocytochemistry was performed using a primary mouse anti-C-terminal nNOS (1095-1289) at a dilution of 1:100 in phosphate-buffered saline (pH 7.4). Grids were washed in phosphate-buffered saline and counterstained with 1% uranyl acetate. Nonspecific background was blocked by incubation with 5% normal goat serum in phosphate-buffered saline at the beginning of the procedure. Specimens were observed in a Zeiss EM-109-T transmission electron microscopy at 80 kv.

Mitochondrial H2O2 Production. H2O2 production was continuously monitored using a Hitachi F-2000 spectrofluorometer (Hitachi) with excitation and emission wavelengths at 315 and 425 nm, respectively.22 The reaction medium, which consisted of potassium phosphate buffer (50 mM) and 50 mM L-valine, was supplemented with 10 mM succinate, 12.5 units/mL horseradish peroxidase, 250 μM p-hydroxyphenyl-acetic acid, and 0.15 mg of mitochondrial protein per mL. NO-dependent H2O2 production was determined as the difference of H2O2 production rate in the presence of 100 μM L-arginine and L-arginine plus 2 mM L-NNMMA. Mitochondrial preparations were supplemented with 1 μM Mn(III)tetrakis(4-benzoic acid) porphyrin (Cayman Chemical, Ann Arbor, MI) to uniform the maximal H2O2 production rate.

Cell Isolation and Culture. Hepatocytes were isolated by collagenase digestion as described previously.23 For culture, P2- and P15-isolated hepatocytes were seeded in 96-well plates (50,000 cells per well) in medium 199 (Gibco-BRL, Invitrogen Life Technologies, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS) and 50 μg/mL gentamicin and were allowed to attach for 4 hours. Cells were synchronized by 20-hour incubation with 2% FCS and 2 hours without FCS. Hepatocyte treatments were performed in medium 199 with 10% FCS for 72 hours. Proliferation was assayed by [3H] thymidine incorporation.11 The last 24 hours, cells were incubated with 0.8 μCi [3H] thymidine/well and harvested; cpm were measured in a liquid scintillation counter (Wallac 1414, Turku, Finland).

Detection of Intracellular ROS. Intracellular ROS were analyzed by flow cytometry, using a 2',7'-dichlorofluorescin diacetate probe.23 The cellular fluorescence intensity was measured after 30 minutes of incubation with 5 μM 2',7'-dichlorofluorescin diacetate by using an Ortho Cytoron Absolute Flow-Cytometer (Johnson & Johnson, Raritan, NY). Propidium iodide (0.005%) was used to detect dead cells. For each analysis, 10,000 events were recorded.

DNA Analysis. For measuring apoptosis, the ploidy determination of hepatocytes was assessed with propidium iodide staining and flow cytometry as described previously.24

Antioxidant Enzyme Activities. Mitochondrial Mn-SOD activity was determined by inhibition of cytochrome c reduction at 550 nm in 50 mM potassium phosphate buffer/0.1 mM EDTA (pH 7.8) at 25°C.25 Catalase and glutathione peroxidase activities in 7,000g supernatants were determined by the decrease in H2O2 absorption at 240 nm (ε240 = 41 μM⁻¹ cm⁻¹),26 or by the oxidation of NADPH at 340 nm (ε340 = 6.22 mM⁻¹ cm⁻¹).27

Reagents. SDS, glycerol, 2-(β-mercaptoethanol), and bromophenol blue were obtained from Bio-Rad (Richmond, CA); SB 202190 was obtained from Calbiochem (San Diego, CA); U0126 was obtained from Cell Signaling Technology; and other chemicals were from Sigma Chemical Co. (St Louis, MO).

Data Analysis. Data are expressed as mean ± SE and were analyzed by ANOVA and Scheffé test. Simple linear
Mitochondrial Maturation in Rat Liver Development. Liver mass continuously increases with age due to cell duplication and hypertrophy (Fig. 1A).\textsuperscript{28} From E19 to adulthood, relative liver weight went from 6.1\% to 3.4\% of body weight and liver growth decreased tenfold (Fig. 1B). Likewise, transition from fetal to adult liver is accompanied by a burst of hepatocyte proliferation in late gestation and in the immediate neonatal period (E19–P2–3), followed by a decrease of proliferation after the first postnatal week.\textsuperscript{13} Instead, mitochondrial biogenesis is persistently active throughout development; mitochondrial protein content increased from 0.13 (E19) to 34 mg per g of liver tissue (P90) (Fig. 1B). To evaluate mitochondrial maturation, enzyme activities were tested in the same conditions. Mitochondria isolated from maximally proliferative liver at E19–P2 retained 40\%–50\% of complex I, II–III, and IV activities of quiescent organelles; mitochondrial ubiquinone (UQ) had a similar pattern of expression—both increased tenfold from fetal to adult stages (Fig. 2A and B). Considering the variations of mitochondrial mass, mtNOS activity per gram of liver tissue enhanced in the adult organ by 500-fold. It is then inferred that, during liver development, (1) matrix NO steady-state concentration raises by sequential increase of mtNOS content and (2) total NO liver production is greatly amplified by the expansion of the mitochondrial pool.

Although it is a nNOS variant,\textsuperscript{3,8} 130-kDa liver mtNOS reacts with both anti-nNOS and anti-iNOS antibodies (see Fig. 2A); eNOS was not detected in liver mitochondria. Interestingly, cytosol immunoblotting with antibodies against nNOS revealed two specific bands: classic nNOS–ca157 kDa and a second band with 130 kDa, the expression of which was inversely modulated. The specificity of the nNOS 130-kDa band variant was validated by immunoprecipitation of mitochondria and cytosolic proteins from P2 liver (see Fig. 2B). Moreover, 130-kDa protein was solely evidenced at early stages while, concomitantly with a higher expression of mtNOS, it disappeared from cytosol after P15. This pattern suggests that cytosol 130-kDa protein is the enzyme translocated to mitochondria during development. Comparatively, cytosolic NOS activity, as contributed by the Ca-dependent isoforms, increased early at birth and remained stable up to adult stage; no significant modulation of the sequential proliferating–differentiating process.\textsuperscript{30} It is surmised that promotion of proliferation requires a controlled inhibition of mitochondrial respiration\textsuperscript{31}; specifically, low complex activities and reduced mitochondrial mass have a negative impact on redox mitochondrial-dependent cell signaling.

Mitochondrial NOS Is Modulated During Development. The increase or decrease of mtNOS content can be an adaptive mechanism to control mitochondrial functions.\textsuperscript{3,9} In this study, liver mtNOS content was clearly modulated during rat development: it was almost undetectable at highly proliferative E19, while it progressively increased after birth, up to quiescent P30–P90 (Fig. 2A); modulation was confirmed by immunoelectron microscopy (Fig. 2E). MtNOS activity paralleled protein expression—both increased tenfold from fetal to adult stages (Fig. 2A and B). Considering the variations of mitochondrial mass, mtNOS activity per gram of liver tissue enhanced in the adult organ by 500-fold. It is then inferred that, during liver development, (1) matrix NO steady-state concentration raises by sequential increase of mtNOS content and (2) total NO liver production is greatly amplified by the expansion of the mitochondrial pool.

Results and Discussion

Mitochondrial Maturation in Rat Liver Development. Liver mass continuously increases with age due to cell duplication and hypertrophy (Fig. 1A).\textsuperscript{28} From E19 to adulthood, relative liver weight went from 6.1\% to 3.4\% of body weight and liver growth decreased tenfold (Fig. 1B). Likewise, transition from fetal to adult liver is accompanied by a burst of hepatocyte proliferation in late gestation and in the immediate neonatal period (E19–P2–3), followed by a decrease of proliferation after the first postnatal week.\textsuperscript{13} Instead, mitochondrial biogenesis is persistently active throughout development; mitochondrial protein content increased from 0.13 (E19) to 34 mg per g of liver tissue (P90) (Fig. 1B). To evaluate mitochondrial maturation, enzyme activities were tested in the same conditions. Mitochondria isolated from maximally proliferative liver at E19–P2 retained 40\%–50\% of complex I, II–III, and IV activities of quiescent organelles; mitochondrial ubiquinone (UQ) had a similar pattern (Table 1). Previous reports showed an increase of the specific activity of a number of oxidative enzymes during the early postnatal development.\textsuperscript{29} Both increasing mitochondrial pool and phenotypic changes may take part in

Table 1. Mitochondrial Enzyme Activities and Ubiquinol Content in Rat Liver Development

<table>
<thead>
<tr>
<th></th>
<th>E19</th>
<th>P2</th>
<th>P15</th>
<th>P30</th>
<th>P90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complexes I–III (nmol/min.mg prot)</td>
<td>170 ± 28*</td>
<td>247 ± 68*</td>
<td>404 ± 54</td>
<td>417 ± 27</td>
<td>441 ± 52</td>
</tr>
<tr>
<td>Complexes II–III (nmol/min.mg prot)</td>
<td>37 ± 7*</td>
<td>48 ± 6*</td>
<td>55 ± 6*</td>
<td>89 ± 9</td>
<td>96 ± 10</td>
</tr>
<tr>
<td>Complex IV (k'/min.mg prot)</td>
<td>5 ± 1*</td>
<td>11 ± 1*</td>
<td>14 ± 1</td>
<td>17 ± 2</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>UQ-9 content (nmol/mg prot) ND</td>
<td>1.1 ± 0.1*</td>
<td>2 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: E, embryonic; P, postnatal; prot, protein; UQ, ubiquinone; ND, not determined.

NOTE: Complexes I–III: NADH-cytochrome c reductase. Complexes II–III: succinate-cytochrome c reductase. Complex IV: cytochrome oxidase. Data are expressed as mean ± SE from 4 – 8 different experiments.

*P < .05 respect to P90.
eNOS expression was detected (Fig. 2B [inset] and D). The selective mtNOS modulation could be related to developmental activities of membrane mitochondrial transporters and chaperone proteins, or to enzyme activation or degradation. It has been reported that arginase and calpain proteases are higher in fetal liver than in adult liver, contributing to low matrix NO levels in fetal hepatocytes by reducing NOS substrate and/or by increasing mtNOS degradation by mitochondrial calpains.

**mtNOS Modulation Correlates With Liver Mitochondrial Hydrogen Peroxide Yield.** Immature mitochondria supplemented with complex III inhibitor antimycin had a noticeably slower \( H_2O_2 \) production rate than adult organelles (Fig. 3A). According to Table 1, respiratory chain activities per unit of mitochondrial protein increases with developmental age, possibly accounting for the decreased \( H_2O_2 \) production rate in less mature mitochondria.

In a physiological setting, supplementation of mitochondria with NO promotes a significant \( O_2^+ \) burst; NO inhibits cytochrome oxidase and \( b-c_1 \) region at complex III and increases the ubisemiquinone radical level that provides the electrons to \( O_2 \). Mitochondrial production of freely diffusible \( H_2O_2 \) depends on superoxide dismutase–catalyzed dismutation of NO-dependent \( O_2^+ \) (reaction [3]):

\[
NO + UQH^- \rightarrow NO^- + UQ^- + H^+ \quad (1)
\]

\[
UQ^- + O_2 \rightarrow UQ + O_2^- \quad (2)
\]

\[
2O_2^- + 2H^+ \xrightarrow{Mn-SOD} H_2O_2 + O_2 \quad (3)
\]
To investigate mtNOS contribution, H$_2$O$_2$ production rate was measured in the presence of L-arginine alone or in addition to L-NMMA. The NO-dependent mitochondrial H$_2$O$_2$ production was undetectable in E17 mitochondria, but thereafter it progressively increased up to P30–P90, indicating a linkage with mtNOS modulation (see Fig. 3A). Furthermore, in embryos low H$_2$O$_2$ yield is cooperatively contributed by low mitochondrial complex activities and UQ content (see reactions [1] and [2] above and Table 1). A parallel increase of mtNOS, UQ, and H$_2$O$_2$ was observed in rat brain in the transit from neuroblast proliferation to neuronal differentiation.11

**Mitochondrial Contributions to Cytosol Liver H$_2$O$_2$ Steady-State Concentration.** Mitochondria is
the main contributor to liver H$_2$O$_2$ steady-state concentration ([H$_2$O$_2$]$_{ss}$); only a small fraction of peroxisome-derived H$_2$O$_2$ appears to escape peroxisomal catalase.\textsuperscript{34}

Depending on matrix NO, in the steady-state condition, mitochondrial H$_2$O$_2$ production equals cytosolic H$_2$O$_2$ utilization by the two main scavenger enzymes, catalase and glutathione peroxidase. NO-dependent [H$_2$O$_2$]$_{ss}$ can be calculated according to the following equation (where \(d[H_2O_2]/dt\) is the rate of L-arginine–dependent H$_2$O$_2$ production, \(k_3\) is the second-order rate constant for the catalase-catalyzed metabolism of H$_2$O$_2$, and \(k_4\) is that for the glutathione peroxidase–driven reaction):\textsuperscript{35}

\[
[H_2O_2]_{ss} = + \frac{d[H_2O_2]}{dt} / k_3 \text{[catalase]} + k_4 \text{[glutathione peroxidase]} \quad (4)
\]

NO-dependent [H$_2$O$_2$]$_{ss}$ was undetectable at E17 and increased by two orders of magnitude from E19 to P90 (\(\approx 10^{-11} \text{ M} \) to \(10^{-9} \text{ M}\)), paralleling the developmental modulation of mtNOS (Fig. 3B and C). Mn-SOD activity was similarly comodulated and well correlated with mtNOS, the net flux of H$_2$O$_2$ into cytosol and the resulted [H$_2$O$_2$]$_{ss}$ (Fig. 3 D and E).

Differential oxidant production was confirmed by flow cytometry (Fig. 4A). The dichlorofluorescin mean fluorescence ratio between P15 and P2 and adult P90 hepatocytes was about 5:15, which is in agreement with that obtained from estimated NO-dependent [H$_2$O$_2$]$_{ss}$ \((\approx 10^{-11}:20)\). Thereby, the contribution of utilized NO to maximal mitochondrial H$_2$O$_2$ production (namely H$_2$O$_2$ index) markedly increased from proliferating to quiescent stages (see Fig. 4A).

These data suggest that most H$_2$O$_2$ comes from mitochondrial metabolism; at P90, NO-dependent H$_2$O$_2$ is similar to that obtained by perfusing adult rat liver.\textsuperscript{35} Therefore, we propose a developmental grading of cytosolic [H$_2$O$_2$]$_{ss}$ as based upon a coordinated increase of mitochondrial complex activities mtNOS and Mn-SOD.

\textbf{Redox Modulation of Hepatocyte Proliferation.} As reported,\textsuperscript{13} in neonatal hepatocytes there is still a synchronized high proliferation rate. We therefore attempted to mimic \textit{ex vivo} the redox modulation of proliferation in P2 and P15 hepatocytes with low and high mtNOS content, respectively. Supplementation of synchronized P2-cultured hepatocytes with H$_2$O$_2$, catalase inhibitor 3-amino-1,2,4-triazole, or L-arginine invariably determined a dose-dependent negative modulation of cell proliferation (Fig. 4B). In contrast, decreasing cell H$_2$O$_2$ levels by controlled treatment with scavengers or NOS inhibitors such as N-acetyl-cysteine (NAC), glutathione, or \(N^G\)-nitro-L-arginine methyl ester increased proliferation rates by up to 30%. At a higher NAC concentration, a similar response was observed in more differentiated and less proliferative P15 hepatocytes with higher [H$_2$O$_2$]$_{ss}$ (see Fig. 4B). In all conditions, cell viability with Trypan blue was 98% and LDH in the culture supernatant was less than 1% of cytosol values, which indicates that at the utilized concentrations the tested compounds were not toxic for hepatocytes. No hypoploid peak representative of apoptosis was observed in the permeabilized P2 hepatocytes in the different conditions (Fig. 4C).

These results confirm that (1) hepatocyte proliferation at different developmental stages depends on a precise tissue H$_2$O$_2$ concentration and (2) in this context, proliferation correlates with liver \textit{in vivo} developmental modulation of mitochondrial activities and mtNOS.

NO may modulate \textit{per se} the expression of cell cycle regulatory proteins,\textsuperscript{19} and it induces cytostasis by inhibition of cyclin D1 or by inhibition of cdc2 (cyclin E and A...
In accordance, NO has antimitogenic effects on cultured hepatocytes.36

**Redox Modulation of Cell Signaling.** In partial hepatectomy or injury,37 mechanisms regulating hepatocyte proliferation rely on MAPK activation by growth factors, but fetal hepatocytes may proliferate in absence of exogenous factors with a constitutive level of MAPK activation.38 Indeed, cyclin D1 is sufficient to promote progression of hepatocytes through G1 restriction point.39 As has been previously reported,13,40 cyclin D1 is up-regulated during liver proliferation (E17–P2) and almost disappears in the quiescent organ; expression of cyclins D2 and D3 followed that of cyclin D1 (Fig. 5A). However, cyclins D2 and D3 would have a lower contribution to adult liver proliferation, as shown during regeneration41 and here after T3 treatment.

ERK activation paralleled cyclin D1 expression while p38 MAPK activation followed an inverse pattern (Fig. 5A and B). Similarly, p38 activity was reported inverse to cyclin D1 content in liver regeneration,13 and transfection with p38-activating kinase MKK6 arrests hepatocyte growth.15 In addition, P2 hepatocytes markedly expressed ERK1/2 upstream kinase phospho–MAPK kinases 1/2 and P90 hepatocytes did not, while phospho–c-Jun N-terminal kinase (a stress-activated kinase related to liver proliferation and apoptosis) was only detectable in the quiescent phenotypes. As referred,42 Phospho–c-Jun N-terminal kinase increase may express both the late increase of oxidant levels and ERK decrease (Fig. 5B). Total MAPKs were not modified during development.

Liver cyclin D1 and phospho-ERKs were inverse to in vivo NO-dependent [H2O2]ss and H2O2 index; instead, this correlation was positive for p38 MAPK (Fig. 5C). Consequently, a high ERK/p38 activity ratio was representative of proliferating phenotypes; a low ERK/p38 ratio was representative of quiescent ones and was related to [H2O2]ss and liver growth. ERK/p38 ratio was considered a determinant of growth and dormancy in human cancer cells; Aguirre-Ghiso et al. showed that modulation of ERK/p38 activity by pharmacological and genetic interventions predicts the in vivo behavior in ≈90% of the examined cell lines.43

The interplay between redox status, signaling, and proliferation was analyzed by exposing P2 hepatocytes to H2O2, antimycin, and NAC, and/or to the ERK inhibitor U0126, p38 inhibitor SB202190, and p38 activator anisomycin. Exposure to H2O2 or to anisomycin markedly reduced cyclin D1 expression, leading P2 cells to the adult liver level. In these conditions, endogenous or exogenous H2O2 decreased ERK/p38 activity ratio, an effect equally prevented by NAC or SB202190 (Fig. 6A and B). Similarly, a marked reduction of ERK/p38 activity ratio by anisomycin or U0126 was clearly associated with the lowest hepatocyte proliferation rate (Fig. 6B and C). Likewise, SB202190 significantly increased cell proliferation by approximately 25%, and SB202190 or NAC also prevented the lowering of the proliferation rate induced by

---

**Fig. 6.** Redox modulation of liver cell signaling. P2 hepatocytes were exposed to H2O2 alone or with 10 μM SB202190 or 0.1 mM NAC (both supplemented 2 hours before H2O2), 200 ng/mL anisomycin, 2 μM antimycin, and 10 μM U0126. (A) Cyclin D1 and MAPKs were detected at 2 hours and 15 minutes, respectively, by immunoblotting (50 μg/lane) in triplicate. (B) P-ERK/P-p38 MAPK ratio (densitometric measurements from three separate experiments) of the different assays is expressed. (C) P2 hepatocytes were incubated in similar conditions as those described in Fig. 4. *Different from basal condition. #Different from respective H2O2 concentration. P < .05. P, postnatal; ERK, extracellular signal–regulated kinase; A, anisomycin; AA, antimycin; SB, SB202190; NAC, N-acetyl-cysteine; MAPK, mitogen-activated protein kinase.
H$_2$O$_2$ ($p < .05$; Fig. 6B and C). These data agree with previous reports that showed that mitochondrial reactive oxygen species initiate phosphorylation of p38 in cardiomyocytes$^{16}$ and that low-level oxidative stress activates p38 and leads to growth arrest in U926 cells, an effect that is inhibited by NAC.$^{15}$

The present results suggest that synchronized increase of mitochondrial activities, mtNOS, and [H$_2$O$_2$]$_{10}$ operates on the balance of liver signaling pathways to drive the transition from proliferation to quiescence. In the same way, we recently reported that a critical reduction of mtNOS activity and [H$_2$O$_2$]$_{ss}$ contribute to tumoral persistent ("embryonic") behavior.$^{44}$ Further studies could confirm the contribution of mitochondrial redox signaling to the modulation of cyclins D2 and D3, which parallels cyclin D1 expression at developmental redox conditions (Fig. 5A and C) and may play a central role in liver development.

Acknowledgment: The authors thank R. Greco for her secretarial assistance, Dr. M. Barbosa for flow cytometry analysis, Dr. L. Schreier and Dr. P. Argibay for technical assistance, and Dr. E. Cadenas for helpful comments.

References