Hypothyroid Phenotype Is Contributed by Mitochondrial Complex I Inactivation Due to Translocated Neuronal Nitric-oxide Synthase*

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Although transcriptional effects of thyroid hormones have substantial influence on oxidative metabolism, how thyroid sets basal metabolic rate remains obscure. Compartmental localization of nitric-oxide synthases is important for nitric oxide signaling. We found liver neuronal nitric-oxide synthase-α (nNOS) subcellular distribution as a putative mechanism for thyroid effects on rat metabolic rate. At low 3,3′,5-triiodo-L-thyronine levels, nNOS mRNA increased by 3-fold, protein expression by one-fold, and nNOS was selectively translocated to mitochondria without changes in other isoforms. In contrast, under thyroid hormone administration, mRNA level did not change and nNOS remained predominantly localized in cytosol. In hypothyroidism, nNOS translocation resulted in enhanced mitochondrial nitric-oxide synthase activity with low O₂ uptake. In this context, NO utilization increased active O₂ species and peroxynitrite yields and tyrosine nitration of complex I proteins that reduced complex activity. Hypothyroidism was also associated to high phospho-p38 mitogen-activated protein kinase and decreased phospho-extracellular signal-regulated kinase 1/2 and cyclin D1 levels. In this context, NO utilization increased active O₂ species and peroxynitrite yields and tyrosine nitration of complex I proteins that reduced complex activity. Hypothyroidism was also associated to high phospho-p38 mitogen-activated protein kinase and decreased phospho-extracellular signal-regulated kinase 1/2 and cyclin D1 levels. In contrast, under thyroid hormone administration, mRNA level did not change and nNOS remained predominantly localized in cytosol. In hypothyroidism, nNOS translocation resulted in enhanced mitochondrial nitric-oxide synthase activity with low O₂ uptake. In this context, NO utilization increased active O₂ species and peroxynitrite yields and tyrosine nitration of complex I proteins that reduced complex activity. Hypothyroidism was also associated to high phospho-p38 mitogen-activated protein kinase and decreased phospho-extracellular signal-regulated kinase 1/2 and cyclin D1 levels. In contrast, under thyroid hormone administration, mRNA level did not change and nNOS remained predominantly localized in cytosol. In hypothyroidism, nNOS translocation resulted in enhanced mitochondrial nitric-oxide synthase activity with low O₂ uptake. In this context, NO utilization increased active O₂ species and peroxynitrite yields and tyrosine nitration of complex I proteins that reduced complex activity. Hypothyroidism was also associated to high phospho-p38 mitogen-activated protein kinase and decreased phospho-extracellular signal-regulated kinase 1/2 and cyclin D1 levels. In contrast, under thyroid hormone administration, mRNA level did not change and nNOS remained predominantly localized in cytosol. In hypothyroidism, nNOS translocation resulted in enhanced mitochondrial nitric-oxide synthase activity with low O₂ uptake. In this context, NO utilization increased active O₂ species and peroxynitrite yields and tyrosine nitration of complex I proteins that reduced complex activity. Hypothyroidism was also associated to high phospho-p38 mitogen-activated protein kinase and decreased phospho-extracellular signal-regulated kinase 1/2 and cyclin D1 levels. In contrast, under thyroid hormone administration, mRNA level did not change and nNOS remained predominantly localized in cytosol. In hypothyroidism, nNOS translocation resulted in enhanced mitochondrial nitric-oxide synthase activity with low O₂ uptake. In this context, NO utilization increased active O₂ species and peroxynitrite yields and tyrosine nitration of complex I proteins that reduced complex activity. Hypothyroidism was also associated to high phospho-p38 mitogen-activated protein kinase and decreased phospho-extracellular signal-regulated kinase 1/2 and cyclin D1 levels. In contrast, under thyroid hormone administration, mRNA level did not change and nNOS remained predominantly localized in cytosol. In hypothyroidism, nNOS translocation resulted in enhanced mitochondrial nitric-oxide synthase activity with low O₂ uptake. In this context, NO utilization increased active O₂ species and peroxynitrite yields and tyrosine nitration of complex I proteins that reduced complex activity. Hypothyroidism was also associated to high phospho-p38 mitogen-activated protein kinase and decreased phospho-extracellular signal-regulated kinase 1/2 and cyclin D1 levels. In contrast, under thyroid hormone administration, mRNA level did not change and nNOS remained predominantly localized in cytosol. In hypothyroidism, nNOS translocation resulted in enhanced mitochondrial nitric-oxide synthase activity with low O₂ uptake. In this context, NO utilization increased active O₂ species and peroxynitrite yields and tyrosine nitration of complex I proteins that reduced complex activity.
amino-2,7'-difluorescein diacetate (DAF-FM), MitoTracker Red 580, SYBR Green, and 39-kDa subunit of Complex I and V1c subunit of Complex IV monoclonal antibodies were from In vitroGen-Molecular Probes. Leukemia virus reverse transcriptase (MMLV-RT) and Taq polymerase were from Promega Corp. (Madison, WI). MAPK antibodies were from Cell Signaling Technology (Beverly, MA). Monoclonal nitrotyrosine antibody was provided by Prof. Álvaro Estévez, University of Alabama at Birmingham. Monoclonal nNOS and polyclonal eNOS antibodies were from BD Transduction Laboratories. Polyclonal nNOS, iNOS, Hsp90, and cyclin D1 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Methimazole was provided by Laboratorios Gador (Buenos Aires, Argentina).

Animals and Treatments—Male Wistar rats (250–300 g) were housed in a temperature-controlled room with food and water ad libitum; National Institutes of Health criteria for animal research were followed after approval by the Ethics Committee of the University Hospital. Rats were divided into five groups (n = 12/group): hypothyroid, (0.02% methimazole (w/v) in drinking water for 28 days); hypothryoid + T3 (after 25 days of methimazole treatment received 15 μg of T3/kg of body weight for 3 days by intraperitoneal injection); hypothryoid + NAME (0.75 mg/ml l-NAME in drinking water in the last 21 days of methimazole treatment); hyperthyroid, (intraperitoneal injection of 60 μg of T3/kg of body weight for 3 days); and control group. Blood samples were collected at the time the animals were killed for estimation of thyrotropin (TSH) level by radioimmunoassay (20).

Basal Metabolic Rate—BMR was measured at 22°C in a non-recirculating open flow system after 30 min of equilibration with an O2-CO2 analyzer in standard temperature and pressure dry conditions (15).

Isolation and Purification of Liver Mitochondria—The livers were excised in ice-cold homogenization medium, and mitochondria were isolated and purified as described (13). Minimal contamination was found (2–3%) by comparing activities of lactate dehydrogenase (cytosolic marker) and succinate-cytochrome c reductase (mitochondrial marker).

RT-PCR—Total liver RNA was extracted with TRIzol, and RT-PCR was performed (21). Primers for β2-microglobulin and NOS isoforms were as described (21–23): for cyclin D1, sense (5’-GGTGATCCCTGACACCAATCT-3’) and antisense (5’-GCTCCAGGACGAAAGCGG-3’). Nested PCR for eNOS isoform (35 cycles) was done with 0.5 μl of PCR product in 30-μl final volume with inner primers, sense (5’-ATGGTGCTGTCTGACATTGGATGAT-3’) and antisense (5’-TTTGCTGACCTTCTTCCAG-3’).

Quantitative Real-time PCR—Real-time nested PCR for nNOS isoform was done with 0.5 μl of a 1/10 dilution of PCR product in 25-μl final volume with inner primers: nNOS, sense (5’-TTCAGCTCATCTGTAACCA-3’) and antisense (5’-GAGCACTGACATTGGCTGAG-3’). Real-time PCR reactions included 0.4 mm dNTPs, 1 mm specific primers, 4 mm MgCl2, 2.5 units of Taq DNA polymerase, and 1:300 SYBR Green. Real-time PCR reactions were performed in DNA Engine Opticon (MJ Research, Inc.) and consisted of an initial denaturing step (94°C for 4 min), followed by 35 cycles (each of 94°C for 1 min, 55°C for 40 s, 72°C for 1 min). Sample quantification was normalized to endogenous β2-microglobulin that was also quantified by real-time PCR following the same protocol as nNOS isoform. Each experiment included a DNA minus control and a standard curve.

Immunoblotting for NOS, Hsp90, and Mitochondrial Protein Nitration—Proteins were electrophoresed on 7.5% SDS-polyacrylamide gel, electrotransferred to polyvinylidene difluoride membranes (13), incubated with anti-nNOS, anti-eNOS, anti-iNOS, anti-Hsp90, and anti-nitrotyrosine antibodies, and detected with the ECL system. Equal loading was controlled with the appropriated subcellular markers. Incubation of the anti-nitrotyrosine antibody with 10 mm nitrotyrosine prior to the membrane incubation was used to ensure the antibody specificity.

NOS Activity in Subcellular Fractions—NOS activity was determined in mitochondrial and cytosolic fractions by conversion of [3H]l-arginine to [3H]l-citrulline (13).

Immunoelectron Microscopy—Purified mitochondria were suspended in 4% paraformaldehyde and 0.5% glutaraldehyde, pH 7.4, for 2 h at 4°C, washed overnight with 0.32 m sucrose at 4°C, and then dehydrated in 70% ethanol and embedded in LR White (13). Ultrathin sections were obtained in 300-mesh nickel grids. Immunocytochemistry was performed using a primary mouse anti-C-terminal nNOS (1095–1289) at a dilution of 1:20 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. Positive control against 39-kDa subunit of complex I (inner membrane marker) and negative control in the absence of a primary antibody were included. Specimens were observed in a Zeiss EM-109-T transmission electron microscope at 80 kV.

Detection of Mitochondrial NO—Mitochondria (1 mg of protein per ml) were incubated in phosphate-buffered saline for 30 min at 37°C with 5% CO2, 10 μM DAF-FM, and 0.5 μM MitoTracker, and fluorescence was measured with an Ortho-Cytomon Absolute Cytometer (Johnson and Johnson) (24).

Mitochondrial O2 Utilization and Electron Transfer Activity—O2 uptake was measured polarographically with a Clark-type electrode (10). To assess NO effects, mitochondria were incubated with 0.3 mm L-arginine (L-Arg) alone or plus 3 mm L-NAME for 5 min at 37°C (10). State 4 O2 uptake was determined with 6 mm malate-glutamate as substrate of complex I and state 3 active respiration by the addition of 0.2 mm adenosine diphosphate (ADP). Complex I activity (NADH: ubiquinone reductase) was measured by the rotenone-sensitive reduction of 50 μM 2,3-dimethoxy-6-methyl-1,4-benzoquinone with 1 mm KCN and 200 μM NADH as electron donor at 340 nm with a Hitachi U3000 spectrophotometer at 30°C. Activity of complexes II-III was determined by cytochrome c reduction at 550 nm. Cytochrome oxidase activity (Complex IV) was determined by monitoring cytochrome c oxidation at 550 nm (ε550 = 21 mm1 cm−1); the reaction rate was measured as the pseudo-first order reaction constant (k’) and expressed as k’/min/mg of protein (13, 14).

Mitochondrial Production of H2O2 and O2−—H2O2 production rate was monitored spectrophotometrically at complexes I or II-III (6 mm malate-glutamate or 10 mm succinate as substrate) in an F-2000 spectrophotometer (Hitachi, Tokyo, Japan) as described (13). To determine O2− at complexes I and II-III, mitochondria were subjected to three freeze/thaw cycles, and SOD-sensitive cytochrome c reduction was measured at 550 nm (0.1 mg of protein/ml and 10 μM SOD to subtract unspecific reduction). Mn-SOD, catalase, and glutathione peroxidase activities were determined in 7,000 g supernatants as described (13).

Liver Cell Isolation and Detection of Intracellular Oxidants—Hepatocytes were isolated by two-step collagenase perfusion (25). Intracellular oxidants and mitochondrial O2− were detected by flow cytometry after incubating hepatocytes in phenol red-free Dulbecco’s modified Eagle’s medium with 5 mm DCFH-DA or 5 μM HE for 30 min at 37°C with 5% CO2.

Blue Native Polyacrylamide Gel Electrophoresis—To separate mitochondrial complexes, Blue Native-PAGE was performed according to Schägger (26). Gels of first dimension were stained with Coomassie Blue and membranes incubated with antibodies against 3-nitrotyrosine. For second-dimension analysis, gel bands, corresponding to the complex I...
region derived from 5-mm-wide lanes, were excised and incubated for 2 h in cathode buffer (50 mM glycine and 7.5 mM imidazole, pH 7) supplemented with 1% SDS and 1% B-mercaptoethanol before electrophoresis on 10%-16.5% Tris/glycine gels (27). Membranes were incubated with antibodies against 3-nitrotyrosine.

Immunoprecipitation—For immunoprecipitations, 500 μg of mitochondrial proteins were incubated with 4 μg of antibodies against Complex I 39-kDa subunit or Complex IV VIc subunit and 30 μg of Protein A/G PLUS-agarose (Santa Cruz) at 4°C; samples were blotted against polyclonal nNOS antibody. Protein loading was controlled by the respective mitochondrial complex antibodies.

Preparation of Whole Liver Homogenates and Immunoblotting for MAPKs and Cyclin D1—To study MAPKs and cyclin D1, liver was homogenized in lysis buffer as described (13). Proteins were separated on 12% SDS-PAGE, and cyclin D1 and MAPKs were detected with specific antibodies.

Metabolic Calculations—All experiments were done at 1 mg of mitochondrial protein per ml (n = 5). Mitochondrial H2O2 steady-state concentration ([H2O2]ss) was calculated according to Ref. 13 as shown in Equation 1.

\[
[H_2O_2]_{ss} = \frac{d[H_2O_2]/dt}{k_1 \times [CAT] + k_2 \times [GPX]} \tag{Eq. 1}
\]

where \(d[H_2O_2]/dt\) is the rate of \(l\)-arginine-dependent \(H_2O_2\) production in \(\text{M} \cdot \text{s}^{-1}\) (Fig. 2A, upper panel), \(k_1 = 4.6 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}\), and \(k_2 = 5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}\). CAT and GPX correspond to catalase and glutathione peroxidase concentrations determined spectrophotometrically as previously described (13).

Mitochondrial \((O_2^-)\) steady-state concentration \(\left([O_2^-]\right)_{ss}\) was calculated from Equations 2 and 3 as follows.

\[
\frac{1}{2}d[H_2O_2]/dt = -d[O_2^-]/dt
\]

\[
d[O_2^-]/dt = k_3[O_2^-][SOD] + k_4[O_2^-][NO] \tag{Eq. 2}
\]

\[
[O_2^-]_{ss} = \frac{1}{2}d[H_2O_2]/dt \tag{Eq. 3}
\]

\(k_3\) and \(k_4\) are, respectively, \(2.3 \times 10^9\) and \(1.9 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}\), and [SOD] was determined spectrophotometrically by inhibition of cytochrome c reduction (13).

Matrix NO \([\text{NO}]_{ss}\) was calculated by the percentage of liver mitochondria state 3 respiratory rate NO-dependent inhibition as previously described (15, 19). \([\text{ONO}^\cdot]\) production rate was calculated as shown in Equation 4.

\[
d[\text{ONO}^\cdot]/dt = k_3[\text{NO}][O_2^-] \tag{Eq. 4}
\]

Statistical Analysis—Data are mean ± S.E. One-way analysis of variance was utilized with post hoc Dunnett test, and regression analysis and significance were accepted at \(p < 0.05\).

RESULTS

At low \(T_3\), Transcriptional Increase of nNOS Enhances mtNOS Content—Thyroid status was discriminated by thyrotropin and BMR measurements (Table 1). In this context, nNOS mRNA, protein expression, and activity were clearly modulated by \(T_3\) levels (Fig. 1, A–E). Likewise, nNOS mRNA expression quantified by RT real-time PCR was >3-fold increased in hypothyroid liver compared with controls (Fig. 1B), whereas eNOS or iNOS mRNA did not change (data not shown). In addition, nNOS became distinctively enhanced in mitochondria (mtNOS), indicating a subsequent import of the overexpressed enzyme to the organelles; these findings were reverted by hormone replacement (Fig. 1C). To corroborate subcellular distribution in the groups, liver fractions were compared. Western blotting confirmed the increase of total nNOS expression represented by liver homogenates and the enrichment of the mitochondrial fraction in the hypothyroid group. Differentially, at high \(T_3\), nNOS expression had results similar to controls, but this condition retained the protein predominantly localized in cytosol (Fig. 1C). This effect was parallel to the increased expression of heat shock protein 90 (Hsp90), one of the most important chaperones associated to nNOS (Fig. 1F). Alternatively, modulation of mtNOS content in the studied groups was validated as well by immunoelectron microscopy with monoclonal nNOS antibodies (Fig. 1D). In agreement, Ca2+-dependent NOS activity was significantly increased in hypothyroid mitochondria but was normal to slightly reduced after \(T_3\) admin-

| TSH (ng/ml) | Control | Hypothyroid | Hypothyroid + T3 | Hyperthyroid |
|------------|---------|-------------|-----------------|-------------|
| Basal metabolic rate (ml of O2/mirg60) | 17 ± 1 | 12 ± 1* | 18 ± 1 | 23 ± 1* |

| Mitochondrial O2 uptake (ngat/mirg of protein) |
|------------------------------------------------|
| State 4 uptake |
| Basal | 14.2 ± 1.3 | 11.5 ± 1.7 | 15.8 ± 1.7 | 16.9 ± 0.3 |
| + 0.3 mm l-Arg | 13.8 ± 0.7 | 9.0 ± 0.5 † | 14.0 ± 1.7 | 14.8 ± 0.6 |
| + 3 mm l-NMMA | 15.6 ± 0.8 | 12.4 ± 2.0 | 14.6 ± 1.8 | 16.2 ± 2 |

| State 3 uptake |
| Basal | 79 ± 6 | 49 ± 3 † | 89 ± 5 | 102 ± 10 |
| + 0.3 mm l-Arg | 72 ± 7 | 46 ± 2 † | 85 ± 5 | 106 ± 10 |
| + 3 mm l-NMMA | 86 ± 8 | 62 ± 4 † | 92 ± 5 | 105 ± 8 |

| Calculated matrix (NO) (nm) |
|-----------------------------|
| Basal | 23 ± 9 | 94 ± 7 | 54 ± 15 | 6 ± 3 |

| Enzyme activities |
|-------------------|
| Complex I (nmole/mirg of protein) | 108 ± 16 | 41 ± 15* | 85 ± 9 | 98 ± 9 |
| Complex II/III (nmole/mirg of protein) | 66 ± 19 | 62 ± 20 | 63 ± 16 | 69 ± 1 |
| Complex IV (k/mirg of protein) | 13.7 ± 0.3 | 12.3 ± 0.2 † | 13.5 ± 0.4 | 14.6 ± 0.4 |

TABLE 1
Oxygen uptake rates depend on thyroid status and nitric oxide utilization

Data are mean ± S.E. of the different groups (n = 5–6 animals/group). O2 uptake rate and complex activities were measured in intact mitochondria and mitochondrial membranes, respectively. †, different from controls; ‡, different from l-NMMA, by analysis of variance and Dunnett post hoc test. To avoid effects of body weight on resting metabolic rate, O2 uptake was corrected to lean body mass and exponentially related to mass0.75. ngat, nanogram atom.
administration, with an opposite cytosolic pattern (Fig. 1E); Ca\(^{2+}\)-independent NOS activity was not detected.

Subcellular nNOS Localization Modulates Mitochondrial Respiration—According to BMR, organelles from hypo- and hyperthyroid groups had the lowest and highest O\(_2\) uptake rates, respectively (Table 1). To discern the effects of mtNOS, mitochondria were supplemented with L-Arg alone or plus NOS inhibitor L-NMMA. The sum of the opposite effects of NOS substrate and inhibitor on basal O\(_2\) utilization determines mtNOS functional activity on respiration (28). Likewise, mtNOS-dependent inhibition of state 3 O\(_2\) uptake was increased ~39% in hypothyroid samples, 18% in controls, and negligible in T\(_3\)-treated mitochondria. In agreement, matrix NO estimated from L-Arg inhibition of O\(_2\) uptake (18) was augmented by 4-fold in hypothyroidism and decreased by a half in hyperthyroidism (Table 1). These results demonstrate that (a) translocated nNOS is functionally active and mitochondria retain ex vivo the cofactors for catalytic activity and (b) NOS confinement to the small mitochondrial compartment amplifies NO effects on O\(_2\) uptake and BMR.

We next examined the contribution of segmental activities to mitochondrial O\(_2\) uptake. Electron transfer rate at complex I was markedly decreased at hypothyroid status solely (~60%), whereas cytochrome oxidase was less inhibited (~11%), and complex II-III activity was not modified (Table 1). No significant thyroid effects on antioxidant Mn-SOD, catalase, or glutathione peroxidase activities were detected.

Thyroid Status Defines Quality and Intensity of Mitochondrial Oxidant Production—In connection with O\(_2\) uptake rate, previous observations proposed a decreased mitochondrial H\(_2\)O\(_2\) yield in rat hypothyroidism and an increased yield in hyperthyroidism (29), though opposite results were reported as well (30, 31). It is shown here that basal production of H\(_2\)O\(_2\) with substrates of complex I (malate-glutamate) or II (succinate) is not essentially modified by thyroid status (Fig. 2A). In contrast, l-Arg enhances the production of mitochondrial active oxygen species from hypothyroid rats, particularly when incubated with malate-glutamate, and L-NMMA prevents its effect. Furthermore, in the presence of L-NMMA, mitochondrial generation of H\(_2\)O\(_2\) in hypothyroidism is below that seen in control and hyperthyroid groups, probably because of its reduced number of functional respiratory chain units. Therefore, mtNOS functional activity on H\(_2\)O\(_2\) production (calculated by the ratio of NO-dependent to maximal H\(_2\)O\(_2\) production in the presence of rotenone (13)), was 33% in hypothyroid mitochondria and 9% in controls. Repercussion of this mitochondrial activity on total cell oxidants in vivo is shown in Fig. 2B. Hypothyroid-isolated hepatocytes exhibited 30% more HE fluorescence and 280% more DCFH fluorescence than T\(_3\)-treated cells, and administration of L-NAME to hypothyroid animals turned oxidant levels down (Fig. 2, B and C).

Considering that mitochondria and cell O\(_2\)\(_2\) and H\(_2\)O\(_2\) steady-state concentrations depend on two O\(_2\)\(_2\)-utilizing reactions as shown in Reactions 1 and 2,
Mn-SOD

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

**REACTION 1**

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

**REACTION 2**

It is then deduced on experimental findings and calculations (Fig. 2C) that (a) at hypothyroid status, increase of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) yields depends on high NO concentration; (b) at high NO, \( \text{O}_2^- \) is also driven to mitochondrial \( \text{ONOO}^- \) formation; (c) consequently, *in vivo* DCFH fluorescence is contributed by both \( \text{H}_2\text{O}_2 \), and \( \text{ONOO}^- \); and (d) in hyperthyroidism, mitochondrial oxidant production should solely depend on \( \text{O}_2 \) uptake rate. In accord with low complex I activity, inhibition of electron transfer with cyanide-myoxothiazole, which mimics NO inhibitory effects on cytochromes c1 and a-a3, markedly enhanced \( \text{O}_2^- \) production rate at complex I in hypothyroidism (Fig. 2D).

**Nitrative Stress in Hypothyroidism**—In relation to expected NO, \( \text{O}_2^- \), and \( \text{ONOO}^- \) yields, mitochondria from hypothyroid animals had higher 3-nitrotyrosine content than organelles from the other groups (Fig. 3A). In particular, complex I was markedly nitrated on tyrosine, whereas complex IV was nitrated to a lesser extent (Fig. 3B). Two-dimensional SDS-PAGE of complex I confirmed an increased nitration of different nuclear and mitochondrial-encoded subunits in hypothyroidism (MW suggests nitration in the region of 75, 50 (ND5), 39 (ND4), 30 (ND1), 22 (PDSW), and 17 kDa (18)). Controls samples showed a constitutive nitration in some of these components, and hyperthyroid samples presented the lowest nitration. In addition, immunoprecipitation of complex I and IV proteins indicated a direct interaction with translocated nNOS, either in control or hypothyroid group (Fig. 3C).

**NOS Inhibitor L-NAME Prevents Low T3-dependent Phenotypic Changes**—To test whether NO inhibition prevents \( \text{T}_3 \) effects on mitochondria, L-NAME was administered to hypothyroid rats. L-NAME neither modified nNOS mRNA (not shown) nor affected nNOS expression or distribution (Fig. 4A). Mitochondrial DAF fluorescence (representa-
matrix NO) rose by 2-fold at hypothyroidism but decreased to only 25% of this value after L-NAME treatment (Fig. 4B). Therefore, at similar thyrotropin levels (80 ng/ml) and mtNOS content, L-NAME prevented NO increase and nitration of mitochondrial proteins (Fig. 4C) and decrease of complex I activity. Accordingly, L-NAME increased BMR of hypothyroid rats up to control values (Fig. 4D), whereas no detectable effects were seen in control animals. These results are consistent with L-NAME prevention of the increase of mitochondrial $\mathrm{O}_2^-$ and oxidants in vivo as shown in isolated hepatocytes (Fig. 2B).

Mitochondrial NOS Activity Contributes to Thyroid-dependent Cell Signaling—Thyroid status governs liver cell proliferation (33). This effect depends on activation of proliferating cascades and D-cyclins that stimulate the progression of cell cycle from G$_0$ to G$_1$. We have previously described the modulation of mtNOS activity and the putative regulation of cell cycle through redox signaling in the sequence of proliferating to quiescent cell stages during rat liver development (13).

In the same way, at the hypothyroid condition, liver P-p38MAPK was markedly expressed, whereas P-ERK1/2 and cyclin D1 mRNA and protein expression were very low (Fig. 5, A and B); the opposite pattern was detected after administration of T$_3$. In this context and regarding mtNOS-dependent redox changes, expression of cyclin D1 correlated with P-ERK1/2/P-p38 ratio that fitted well with experimentally measured mitochondrial $d[H_2O_2]/dt\div d[NO]/dt$ (Fig. 5C), or with calculated $d[H_2O_2]/dt\div d[ONOO^-]/dt$ ratios (not shown). Interestingly, concomitant administration of L-NAME to hypothyroid rats had the same effects on signaling as administration of T$_3$, without changing thyroid status.

**DISCUSSION**

This study shows, for the first time, that thyroid hormones modulate mRNA expression and subcellular distribution of nNOS. As reported for eNOS subcellular traffic (34, 35), the translocation of nNOS to mitochondria could be influenced by regulation of posttranslational changes found in mitochondrial nNOS, (N-acetylation, Ser-1412 phosphorylation) or by the turnover of cytosol "anchoring" proteins, like Hsp90 (Fig. 1F), dystrophin (36), or caveolin-1 (37).
The notion that spatial confinement is essential to NOS signaling (6, 38) is supported here by differential modulation of O$_2$ uptake and nNOS distribution, depending on thyroid status; this notion could be extended to other cell adaptive responses (15, 39). It is then surmised that mitochondrial biogenesis by cGMP-dependent activation of peroxisome proliferator-activated receptor-γ coactivator 1α that stimulates the transcription of nuclear-encoded mitochondrial proteins (47). It is worth noting that the administration of l-NAME completely prevents the phenotypic changes induced by hypothyroidism (low BMR, decreased complex I activity, increased protein nitration) without changing hormonal status. Considering that l-NAME did not modify nNOS expression or subcellular distribution, its effects likely rely on the inhibition of NOS activity within mitochondria.

In recent years, some reports indicated mitochondrial redox contribution to the activation of MAPK cascades (48). In agreement, higher levels of phosphorylated p38MAPK could be consistent with kinase activation by high oxidant levels or by ONOO$^-$. Itself produced in hypothyroidism (13, 49). Currently, P-p38 participates in cell cycle arrest and inhibition of cell proliferation, a hallmark of hypothyroidism (1), whereas low oxidative stress and low ONOO$^-$ in hyperthyroidism are associated here with liver ERK1/2 activation and cyclin D1 expression (13), a hallmark of tissue proliferation. A similar effect of l-NAME or T$_3$ in turning hypothroid cell signaling back to control status indicates that differential ERK1/2/P-p38MAPK ratio and expression of cyclin D1 should depend not on thyroid hormones themselves but on the relative production of mitochondrial oxidants, NO and ONOO$^-$. at the different T$_3$ levels. T$_3$-dependent targeting of nNOS to mitochondria provides a new insight into the pathophysiology of hypothyroid and hyperthyroid syndromes. The presented mechanisms may gain importance in other situations associated with complex I dysfunction, like Parkinson disease.

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REFERENCES

1. Moro, L., Marra, E., Capuano, F., and Greco, M. (2004) Endocrinology 145, 5121–5128
2. Scanlan, T. S., Suchland, K. L., Hart, M. E., Chiellini, G., Huang, Y., Kruzeich, P. J., Frascarelli, S., Crossley, D. A., Bunzow, J. R., Ronca-Testoni, S., Lin, E. T., Hatton, D., Zucchi, R., and Grandy, D. K. (2004) Nat. Med. 10, 638–642
3. Pillar, T. M., and Seitz, H. J. (1997) Eur. J. Endocrinol. 136, 231–239
4. Liggett, S. B. (2004) Nat. Med. 10, 582–583
5. Toft, A. D. (1994) New Engl. J. Med. 331, 171–180
6. Barouch, L. A., Harrison, R. W., Skaf, M. W., Rosas, G. O., Cappola, T. P., Kopeiissi, Z. A., Hobi, I. A., Lemmon, C. A., Burnett, A. L., O’Rourke, B., Rodriguez, E. R., Huang, P. L., Lima, J. A., Berkowitz, D. E., and Hare, J. M. (2002) Nature 416, 337–339
7. Fulton, D., Rabitt, R., Zeolner, S., Fontana, J., Acevedo, L., McCabe, T. J., Iwakiri, Y., and Sessa, W. C. (2004) J. Biol. Chem. 279, 30349–30357
8. Giulivi, C., Poderoso, J. J., and Boveris, A. (1998) J. Biol. Chem. 273, 11038–11043
9. Ghafourifar, P., and Cadenas, E. (2005) Trends Pharmacol. Sci. 26, 190–195
10. Carreras, M. C., Peralta, J. G., Converso, D. P., Finocchietto, P. V., Rebagliati, I., Zaninovich, A. B., and Poderoso, J. J. (2001) Am. J. Physiol. 281, H2282-H2288
11. Ghafourifar, P., Schenk, U., Klein, S. D., and Richter, C. (1999) J. Biol. Chem. 274, 31185–31188
12. Elffering, S. L., Haynes, V. L., Traaseth, N. J., Ettl, A., and Giulivi, C. (2004) Am. J. Physiol. 286, H22-H29
13. Carreras, M. C., Converso, D. P., Lorenti, A. S., Sarbich, M., Levinson, D. M., Jaitovich, A., Arrizco Arciuch, V. G., Galli, S., and Poderoso, J. J. (2004) Hepatology 40, 2027–2032.

FIGURE 5. Liver cell signaling depends on thyroid-dependent mitochondrial active species. A, on the left, Western blots show differential activation of MAPKs of liver homogenates at different T$_3$ levels; on the right, densitometry of five separate experiments (for simplification, P-ERK is the sum of P-ERK1 and P-ERK2, at equal loading control). B, RT-PCR for cyclin D1 is compared with β2-microglobulin, and Western blotting of cyclin D1 is included (n = 5), C, as a marker of liver proliferation rate at different thyroid status, cyclin expression correlates with ERK1/2/P-p38 ratio (left), which fits with experimentally measured mitochondrial dH$_2$O$_2$/dNO$_2$/dO$_2$/dNO ratio (Figs. 2A and 1C); dH$_2$O$_2$/dwt was measured with 6 mM malate-glutamate as substrate. *, p < 0.05 compared with control by analysis of variance. Regressions are significant at p < 0.05 level.
157–166

14. Riobo, N. A., Melani, M., Sanjuan, N., Fiszman, M. L., Gravielle, M. C., Carreras, M. C., Cadenas, E., and Poderoso, J. J. (2002) J. Biol. Chem. 277, 42447–42455

15. Peralta, J. G., Finocchietto, P. V., Converso, D., Schopfer, F., Carreras, M. C., and Poderoso, J. J. (2003) Am. J. Physiol. 284, H2375–H2383

16. Valdez, L. B., Zaehornys, T., Alvarez, S., Bustamante, J., Costa, L. E., and Boveris, A. (2004) Mol. Aspects Med. 25, 49–59

17. Brookes, P. S., Kraus, D. W., Shiva, S., Doeller, J. E., Barone, M. C., Patel, R. P., Lancaster, J. R., Jr., and Darley-Usmar, V. (2003) J. Biol. Chem. 278, 31603–31609

18. Poderoso, J. J., Carreras, M. C., Lisdero, C., Riobo, N., Schopfer, F., and Boveris, A. (1996) Arch. Biochem. Biophys. 328, 85–92

19. Poderoso, J. J., Lisdero, C., Schopfer, F., Riobo, N., Carreras, M. C., Cadenas, E., and Boveris, A. (1999) J. Biol. Chem. 274, 37709–37716

20. Valdez, L. B., Zaobornyj, T., Alvarez, S., Bustamante, J., Costa, L. E., and Boveris, A. (2004) Mol. Aspects Med. 25, 49–59

21. Brookes, P. S., Kraus, D. W., Shiva, S., Doeller, J. E., Barone, M. C., Patel, R. P., Lancaster, J. R., Jr., and Darley-Usmar, V. (2003) J. Biol. Chem. 278, 31603–31609

22. Poderoso, J. J., Carreras, M. C., Lisdero, C., Riobo, N., Schopfer, F., and Boveris, A. (1996) Arch. Biochem. Biophys. 328, 85–92

23. Poderoso, J. J., Lisdero, C., Schopfer, F., Riobo, N., Carreras, M. C., Cadenas, E., and Boveris, A. (1999) J. Biol. Chem. 274, 37709–37716

24. Valdez, L. B., Zaobornyj, T., Alvarez, S., Bustamante, J., Costa, L. E., and Boveris, A. (2004) Mol. Aspects Med. 25, 49–59

25. Brookes, P. S., Kraus, D. W., Shiva, S., Doeller, J. E., Barone, M. C., Patel, R. P., Lancaster, J. R., Jr., and Darley-Usmar, V. (2003) J. Biol. Chem. 278, 31603–31609

26. Poderoso, J. J., Carreras, M. C., Lisdero, C., Riobo, N., Schopfer, F., and Boveris, A. (1996) Arch. Biochem. Biophys. 328, 85–92

27. Poderoso, J. J., Lisdero, C., Schopfer, F., Riobo, N., Carreras, M. C., Cadenas, E., and Boveris, A. (1999) J. Biol. Chem. 274, 37709–37716

28. Valdez, L. B., Zaobornyj, T., Alvarez, S., Bustamante, J., Costa, L. E., and Boveris, A. (2004) Mol. Aspects Med. 25, 49–59

29. Brookes, P. S., Kraus, D. W., Shiva, S., Doeller, J. E., Barone, M. C., Patel, R. P., Lancaster, J. R., Jr., and Darley-Usmar, V. (2003) J. Biol. Chem. 278, 31603–31609

30. Poderoso, J. J., Carreras, M. C., Lisdero, C., Riobo, N., Schopfer, F., and Boveris, A. (1996) Arch. Biochem. Biophys. 328, 85–92

31. Poderoso, J. J., Lisdero, C., Schopfer, F., Riobo, N., Carreras, M. C., Cadenas, E., and Boveris, A. (1999) J. Biol. Chem. 274, 37709–37716

32. Valdez, L. B., Zaobornyj, T., Alvarez, S., Bustamante, J., Costa, L. E., and Boveris, A. (2004) Mol. Aspects Med. 25, 49–59

33. Brookes, P. S., Kraus, D. W., Shiva, S., Doeller, J. E., Barone, M. C., Patel, R. P., Lancaster, J. R., Jr., and Darley-Usmar, V. (2003) J. Biol. Chem. 278, 31603–31609

34. Poderoso, J. J., Carreras, M. C., Lisdero, C., Riobo, N., Schopfer, F., and Boveris, A. (1996) Arch. Biochem. Biophys. 328, 85–92

35. Poderoso, J. J., Lisdero, C., Schopfer, F., Riobo, N., Carreras, M. C., Cadenas, E., and Boveris, A. (1999) J. Biol. Chem. 274, 37709–37716

36. Valdez, L. B., Zaobornyj, T., Alvarez, S., Bustamante, J., Costa, L. E., and Boveris, A. (2004) Mol. Aspects Med. 25, 49–59

37. Brookes, P. S., Kraus, D. W., Shiva, S., Doeller, J. E., Barone, M. C., Patel, R. P., Lancaster, J. R., Jr., and Darley-Usmar, V. (2003) J. Biol. Chem. 278, 31603–31609

38. Poderoso, J. J., Lisdero, C., Schopfer, F., Riobo, N., Carreras, M. C., Cadenas, E., and Boveris, A. (1999) J. Biol. Chem. 274, 37709–37716

39. Valdez, L. B., Zaobornyj, T., Alvarez, S., Bustamante, J., Costa, L. E., and Boveris, A. (2004) Mol. Aspects Med. 25, 49–59

40. Brookes, P. S., Kraus, D. W., Shiva, S., Doeller, J. E., Barone, M. C., Patel, R. P., Lancaster, J. R., Jr., and Darley-Usmar, V. (2003) J. Biol. Chem. 278, 31603–31609

41. Poderoso, J. J., Lisdero, C., Schopfer, F., Riobo, N., Carreras, M. C., Cadenas, E., and Boveris, A. (1999) J. Biol. Chem. 274, 37709–37716

42. Valdez, L. B., Zaobornyj, T., Alvarez, S., Bustamante, J., Costa, L. E., and Boveris, A. (2004) Mol. Aspects Med. 25, 49–59

43. Brookes, P. S., Kraus, D. W., Shiva, S., Doeller, J. E., Barone, M. C., Patel, R. P., Lancaster, J. R., Jr., and Darley-Usmar, V. (2003) J. Biol. Chem. 278, 31603–31609

44. Valdez, L. B., Zaobornyj, T., Alvarez, S., Bustamante, J., Costa, L. E., and Boveris, A. (2004) Mol. Aspects Med. 25, 49–59

45. Brookes, P. S., Kraus, D. W., Shiva, S., Doeller, J. E., Barone, M. C., Patel, R. P., Lancaster, J. R., Jr., and Darley-Usmar, V. (2003) J. Biol. Chem. 278, 31603–31609