Modulation of liver mitochondrial NOS is implicated in thyroid-dependent regulation of O₂ uptake

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Carreras, María Cecilia, Jorge G. Peralta, Daniela P. Converso, Paola V. Finocchietto, Inés Rebagliati, Ángel A. Zaninovich, and Juan José Poderoso. Modulation of liver mitochondrial NOS is implicated in thyroid-dependent regulation of O₂ uptake. Am J Physiol Heart Circ Physiol 281: H2282–H2288, 2001.—Changes in O₂ uptake at different thyroid status have been explained on the basis of the modulation of mitochondrial enzymes and membrane biophysical properties. Regarding the nitric oxide (NO) effects, we tested whether liver mitochondrial nitric oxide synthase (mtNOS) participates in the modulation of O₂ uptake in thyroid disorders. Wistar rats were inoculated with 400 μCi ¹³¹I (hypothyroid group), 20 μg thyroxine (T₄)/100 g body wt administered daily for 2 wk (hyperthyroid group) or vehicle (control). Basal metabolic rate, mitochondrial function, and mtNOS activity were analyzed. Systemic and liver mitochondrial O₂ uptake and cytochrome oxidase activity were lower in hypothyroid rats with respect to controls; mitochondrial parameters were further decreased by L-arginine (−42 and −34%, P < 0.05), consistent with 5- to 10-fold increases in matrix NO concentration. Accordingly, mtNOS expression (75%) and activity (260%) were selectively increased in hypothyroidism and reverted by hormone replacement without changes in other nitric oxide isoforms. Moreover, mtNOS activity correlated with serum 3,5,3'-triiodothyronine (T₃) and O₂ uptake. Increased mtNOS activity was also observed in skeletal muscle mitochondria from hypothyroid rats. Therefore, we suggest that modulation of mtNOS is a substantial part of thyroid effects on mitochondrial O₂ uptake.

hypothyroidism; oxygen uptake regulation; hyperthyroidism

BASAL METABOLIC RATE and systemic O₂ uptake are representative parameters of the energy cost of living for endotherms and exotherms. At thermoneutral environment, most of the resting O₂ uptake is consumed in the mitochondrial synthesis of ATP to provide energy to cell pumps and biosynthetic metabolism. However, some energy is dissipated to counteract the proton back flow not involving ATP synthesis in the mitochondrial inner membrane (proton leak), partly depending on the existence of proton motive force (electrochemical potential difference from protons across the inner membrane) and partly as an intrinsic property of mitochondrial membranes (5, 19).

Resting O₂ uptake and basal metabolic rate are typically regulated by the thyroid state; hypothyroidism and hyperthyroidism are opposite conditions associated to decreased and increased basal O₂ uptake, respectively (16, 22). Thyroid hormone effects are exerted on the mitochondria of specific target tissues such as the liver and skeletal muscle, the most important O₂ consumers, whereas oxidative metabolism of other organs such as the brain is not affected (26).

The action of thyroid hormone on respiratory function has been considered to be operating at two levels: by altering mitochondrial inner membrane composition and permeability and by influencing respiratory gene expression. Changes in properties and composition of mitochondrial membranes, particularly in cardiolipin content, lead to variations of redox enzyme activities (19, 29) and in proton leak (5). Activation or inhibition of nuclear gene transcription by 3,5,3'-triiodothyronine (T₃) bound to nuclear thyroid receptors has been related to transcriptional changes in the expression of some genes encoding components of the respiratory chain like cytochrome c₁ and F₁-ATPase (22). Nevertheless, changes on the nuclear signaling pathway or in the expression of redox enzymes are not consistently found (22).

On the other hand, in the last years, it has been reported by different research groups (7, 8, 23, 33) that nitric oxide (NO) regulates mitochondrial O₂ uptake by a high affinity and reversible binds to the Cu²⁺ center of cytochrome oxidase. Inhibition of cytochrome oxidase activity depends on the O₂-to-NO ratio (3) and represents an important adaptive response to changes in blood flow (4, 24). Accordingly, activation of endothelial NO synthase (eNOS) by bradykinin induces a prompt decrease in myocardial O₂ uptake (24). The recent finding of a distinct NOS in rat liver mitochondria (mtNOS) by independent groups (12, 13) added a new perspective on the regulation of mitochondrial O₂ uptake in thyroid disorders.

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drial functions. The mtNOS releases NO vectorially into the mitochondrial matrix, a cellular differentiated compartment; in this context, effects of NO on the components of the electron transfer chain, particularly on cytochrome oxidase, are amplified. By this mean, mtNOS may finely modulate mitochondrial O$_2$ uptake in the presence of its substrate l-arginine (l-Arg) (4).

Recently, a report was focused on the regulation of NO expression by thyroid hormones (11). Furthermore, we hypothesized here that, in target tissues like the liver, thyroid-dependent regulation of O$_2$ uptake selectively involves the modulation of mtNOS. Likewise, the thyroid-dependent variations in basal metabolic rate should include the correlative percentage of NO-dependent inhibition of mitochondrial O$_2$ uptake.

**EXPERIMENTAL PROCEDURES**

**Experimental design.** Wistar rats (230–280 g body wt) were divided in three major groups: a hypothyroid group that was injected intraperitoneally with a single dose of $^{131}$I (400 $\mu$Ci/100 g body wt), a hyperthyroid group that was daily injected subcutaneously with $g$ thyroxine (T$_4$)/100 g body wt over 2 wk, and the control group. The hypo- and hyper-thyroid state was confirmed by T$_3$ blood analysis after 30 and 15 days of treatment. To return rats to the euthyroid state, a subgroup of hypothyroid rats was injected subcutaneously with T$_4$ (2 $\mu$g/100 g body wt) for 3–6 consecutive days.

**Systemic O$_2$ consumption.** Whole animal O$_2$ consumption was measured in an open circuit. Room air was drawn into the chamber and released through the outflow opening. The effluent gas from the chamber was sampled with a vacuum pump, drawn through anhydrous CaSO$_4$ and into an O$_2$ analyzer (OM-14 Beckman Instruments), and into a CO$_2$ analyzer (Godart Statham; NV Bilthove, Holland) set in series. Consumption was calculated from the measured flux through the chamber, the expired fractions of effluent O$_2$ and CO$_2$ in room air, the air temperature, and barometric pressure. Expired gases were corrected to standard temperature, pressure, and dry weight. Measurements were done by triplicate after a 30-min habituation period. O$_2$ uptake was corrected to lean body mass [expressed as ml O$_2$/min$\cdot$body mass$^{0.73}$] to avoid the effects of body mass variations of the studied groups on resting metabolic rates, as previously described (20).

**Isolation and purification of rat liver mitochondria.** Excised liver tissue samples (mean weight 4 g) were placed in an ice-cold homogenization medium consisting of 0.23 M mannitol, 70 mM sucrose, 10 mM Tris$\cdot$HCl, 1 mM EDTA, 5 $\mu$g aprotinin, 100 $\mu$g/ml phenylmethylsulfonyl fluoride with 0.5% bovine serum albumin, pH 7.4. Mitochondria from the gastrocnemius muscle were isolated in Chappell-Perry buffer (31) at 4°C for 10 min. The supernatant was decanted and centrifuged at 7,000 $g$ for 10 min (2). The mitochondrial pellet was further purified using Percoll gradient to completely remove contaminating organelles and broken mitochondria (18). Briefly, mitochondria were resuspended in 30% Percoll in 225 mM mannitol, 70 mM sucrose, 1 mM EGTA, 25 mM HEPES, and 0.1% bovine serum albumin (MSHE). The solution was spun at 95,000 $g$ for 30 min, and the ring with a density of 1.025–1.075 g/ml was collected and washed twice with MSHE to remove Percoll, twice with 150 mM KCl to remove attached proteins like arginase, followed by two washings with MSHE without BSA. Mitochondria were finally resuspended to 30 mg of protein/ml.

**Mitochondrial respiratory activities and respiratory control ratio.** Oxygen uptake was determined polarographically with a Clark-type electrode placed in a 3-ml chamber at 30°C, in reaction medium consisting of 0.23 M mannitol, 70 mM sucrose, 30 mM Tris$\cdot$HCl, 4 mM MgCl$_2$, 5 mM Na$_2$HPO$_4$, 0.3 M KH$_2$PO$_4$, and 1 mM EDTA, pH 7.4, saturated with room air (225 $\mu$M O$_2$) with 0.5–1 mg mitochondria protein/ml. Oxygen uptake was determined with 6 mM malate-glutamate as substrates in the presence (state 3) or the absence (state 4) of phosphate acceptor (0.2 mM ADP). Oxygen uptake was expressed in nanogram atoms oxygen per minute per milligram of protein. Respiratory control rate was calculated as state 3/state 4 respiration rate. The P/O ratio was calculated as the ratio of nanomoles of added ADP per nanogram atoms of O$_2$ utilized during state 3 (10). Supplementation of 0.3 mM l-Arg with or without 3 mM $^{N^\bullet,N^\bullet}$-nitro-l-arginine (l-NNMA) to the incubation medium was performed immediately before the mitochondrial protein (1 mg/ml) addition. As previously reported (12), l-NMMA resulted particularly suitable for the analysis of mtNOS-dependent effects on mitochondrial O$_2$ uptake.

**Cytochrome oxidase activity.** Enzyme activity was determined by monitoring the oxidation of reduced cytochrome c in a Hitachi U-3000 spectrophotometer at 550 nm; $E_{550} = 21$ mM$^{-1}\cdot$cm$^{-1}$ (31). Cytochrome c was reduced with potassium ascorbate that was removed afterward by eluting through a Sephadex G-25 column with potassium phosphate buffer (10 mM), pH 7.4. Cytochrome oxidase activity was determined in the mitochondrial (50 $\mu$g/ml) preincubated for 2 min with or without NOS substrate (0.1 mM l-Arg) or inhibitor (1 mM aminoguanidine). The reaction was initiated by addition of 50 $\mu$M cytochrome c, and the rate of cytochrome c oxidation was determined as a pseudo-first-order reaction constant ($k'$) [expressed as $k'$ (min$^{-1}\cdot$mg protein$^{-1}$)].

**Mitochondrial protein determination.** Protein concentration was determined by the Lowry assay using bovine serum albumin as standard.

**Western blot analysis.** Proteins (50 $\mu$g/lane) were separated by electrophoresis on 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with a rabbit polyclonal IgG anti-mouse inducible NOS (iNOS) antibody (1:500) or anti-eNOS (1:1,000). Immunoreactive proteins were detected using a chemiluminescence system. Membranes were blotted with a donkey anti-rabbit IgG (1:3,000) conjugated to horseradish peroxidase, followed by detection of immunoreactive proteins by a chemiluminescence system. Liver homogenates of lipopolysaccharide-treated rats were used as a iNOS-positive control, and rat diaphragm homogenates were used as a eNOS-positive control (1). Quantification of bands was performed by digital image analysis using a Hewlett-Packard scanner and Totallab analyzer software (Nonlinear Dynamics, Biodynamics).

**NOS activity.** NOS activity was determined by the conversion of L-[H]$^3$H]arginine to L-[H]$^3$H[citrulline (21). The reaction medium consisted of $0.1 \mu$M L-[H]$^3$H]arginine, 50 $\mu$M L-arginine, 0.1 mM NADPH, 0.3 mM CaCl$_2$, 0.1 $\mu$M calmodulin, 10 $\mu$M BH$_4$, 1 $\mu$M FAD, $\mu$M flavinmononucleotide, 50 mM L-Valine in 50 mM potassium phosphate buffer (pH 7.5), and 0.1 mg of mitochondrial protein. The radiolabel present in the NOS inhibitor blank (2 mM aminoguanidine for liver mtNOS and 2 mM l-NMMA for eNOS and skeletal muscle mtNOS) was subtracted from that present in the other incubations to leave the radiolabel corresponding to NOS-dependent citrulline formation.
Serum T₃. Hormone levels were measured by RIA using a commercial kit (Diagnostic Products; Los Angeles, CA). Corrections were made for the difference in nonspecific binding derived from different plasma binding between humans and rat as previously described (34).

Statistical analysis. Data are expressed as means ± SE. To assess statistical differences, the data were analyzed by ANOVA and the Dunnett’s test or unpaired Student’s t-test as corresponded. Data correlations were analyzed by simple linear regression. Statistical significance was accepted when \( P < 0.05 \).

Materials. SDS, glycerol, 2-(β-mercaptoethanol), and bromophenol blue were obtained from Bio-Rad (Richmond, CA). L-[³H]arginine was from NEN-DuPont (Boston, MA). The antibody against iNOS was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Western blotting detection system and Hybond-ECL membranes were purchased from Amersham Pharmacia Biotech. Other chemicals and biochemicals were purchased from Sigma (St. Louis, MO).

RESULTS

Thyroid status regulates systemic and mitochondrial O₂ uptake. Systemic O₂ consumption was decreased in the hypothyroid group by −30%, and it was partially reverted to −13% of control values by T₄ treatment, whereas the hyperthyroid rats showed an increase in O₂ uptake of ~20% (Table 1). Accordingly, state 4 (without ADP) and state 3 (with ADP) respiratory rates of liver mitochondria from hypothyroid rats were decreased, compared with control rat mitochondria, by −16% (nonsignificant) and −30% \( (P < 0.05) \), respectively, and returned back to control values after replacement with T₄ treatment. In opposite, in hyperthyroid rats, state 4 and 3 rates significantly increased 47 and 35%, respectively \( (P < 0.05) \). No differences were found in respiratory control rate or P/O ratio, supporting the efficiency of the oxidative phosphorylation of the respiratory chain in all the studied conditions.

MtNOS activity is modulated by thyroid hormone state. The activity of mtNOS was markedly modulated by thyroid status. Hypothyroid rats showed two- to fourfold increased L-[³H]citrulline production by liver mitochondria that were reverted by T₄ treatment (Fig. 1A). In accord, mtNOS activity was markedly diminished in the hyperthyroid group with respect to the control one.

The increase in liver mtNOS activity in hypothyroidism was associated with an increase in the expression of the enzyme as evaluated by Western blot analysis.

| Table 1. Regulation of systemic and liver mitochondrial oxygen uptake by thyroid status |
|------------------|------------------|------------------|------------------|------------------|
| Serum T₃ levels, ng/dl | Control | Hypothyroidism | Hypothyroidism + T₄ | Hyperthyroidism |
| Systemic O₂ uptake, ml O₂/min·kg body mass³/₂ | 62 ± 5 | 39 ± 3* | 61 ± 12 | 118 ± 10* |
| Mitochondrial O₂ uptake, ngat O₂/min·mg protein⁻¹ | 26 ± 1 | 16 ± 2* | 20 ± 1 | 30 ± 1 |
| State 4 | 19 ± 2 | 16 ± 1 | 20 ± 2 | 28 ± 2* |
| State 3 | 121 ± 9 | 85 ± 7* | 118 ± 13 | 163 ± 13* |
| RCR | 6.4 ± 0.4 | 5.9 ± 0.5 | 5.9 ± 0.4 | 5.8 ± 0.3 |
| P/O | 3.1 ± 0.1 | 3.0 ± 0.1 | 2.9 ± 0.1 | 2.9 ± 0.1 |

Data are means ± SE from 6 to 12 samples. Respiratory control rate (RCR) is defined as the state 3/state 4 respiration rates and P/O as ADP added/O₂ consumed; T₄, thyroxine; T₃, 3,5,3'-thyronine. *P < 0.05, with respect to control samples by ANOVA and Dunnett’s test.

Fig. 1. Modulation of liver mitochondrial nitric oxide synthase (NOS) (mtNOS) activity and expression by thyroid status. A: NOS activity from purified rat liver mitochondria was measured by the conversion of L-[³H]arginine to L-[³H]citrulline. Each group consisted of 6–10 samples measured by duplicate. Con, control; Hyp, hypothyroidism; Hyper, hyperthyroidism. B: liver mtNOS expression. Densitometry is shown as means ± SE from 6 independent experiments. Bottom: representative Western blot; C corresponds to inducible NOS (iNOS)-positive control. T₄, thyroxine. *P < 0.05 by ANOVA and Dunnett’s test.
with anti-iNOS antibodies (13); densitometry analysis of the bands showed a 75\% increase in hypothyroidism with respect to controls, which was reverted by T\(_4\) replacement (Fig. 1B). No significant changes were observed in the hyperthyroid samples.

Interestingly, similar effects were observed in gastrocnemius mitochondria from the four studied groups (Fig. 2). Basal specific activity of skeletal muscle mtNOS was 25\% of the liver and increased by the same percentage in hypothyroidism (270\%).

To study the specificity of the effects of thyroid hormones on the activity and/or expression of liver mtNOS, we also measured NO activity in the supernatant from a centrifugation of 7,000 \text{g} where endothelial NOS is detected by Western blot analysis (Fig. 3). No significant variations were observed in either the activity or the expression of eNOS in any of the studied groups; in addition, cytosolic iNOS was not detected by immunoblotting (data not shown).

**Rat liver mtNOS activity and O\(_2\) uptake in hypothyroidism.** To relate increased mtNOS activity to hypo- thyroid-induced inhibition of O\(_2\) uptake, we measured mitochondrial activities in the presence of L-Arg (0.1–0.3 mM) (Table 2). Addition of L-Arg inhibited the state 3 O\(_2\) uptake more markedly in hypothyroid organelles than in control ones (–23\%, \(P < 0.05\), and –8\%, respectively); in the same conditions, supplementation of the preparations with 10-fold higher concentrations of NOS inhibitor L-NMMA proportionally restored mitochondrial respiratory rates in both groups (Table 2). These variations are relevant, taking into account that effects of NO are more effective at low physiological O\(_2\)/NO ratios than at experimentally used 220 \(\mu\text{M} \text{O}_2\) (3).

Accordingly, systemic O\(_2\) uptake, state 3 mitochondrial respiratory rate, and \(L-[^3H]\)citrulline production significantly correlated with serum T\(_3\) levels (\(r: 0.87\), \(P < 0.0001\); \(r: -0.84\), \(P < 0.0001\); and \(r: -0.73\), \(P = 0.003\), respectively) (Fig. 4, A–C); in addition, mtNOS activity correlated well with state 3 respiratory rate (\(r: -0.77\), \(P < 0.0001\)) (Fig. 4D) and with systemic O\(_2\) uptake (\(r: -0.80\), \(P < 0.0001\)).

**Table 2. Effect of L-arginine on mitochondrial oxygen uptake and cytochrome oxidase activity in hypothyroidism**

<table>
<thead>
<tr>
<th>State 4, ngat O(_2)/min (1\text{-mg protein}^{-1})</th>
<th>Control</th>
<th>Hypothyroidism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without l-Arg</td>
<td>19 ± 2</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>+0.3 mM L-Arg</td>
<td>18 ± 2</td>
<td>13 ± 1*</td>
</tr>
<tr>
<td>+3 mM L-NMMA</td>
<td>19 ± 2</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>State 3, ngat O(_2)/min (1\text{-mg protein}^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without l-Arg</td>
<td>122 ± 9</td>
<td>92 ± 7*</td>
</tr>
<tr>
<td>+0.3 mM L-Arg</td>
<td>113 ± 11</td>
<td>71 ± 2††</td>
</tr>
<tr>
<td>+0.3 mM L-Arg +3 mM l-NMMA</td>
<td>117 ± 10</td>
<td>85 ± 1</td>
</tr>
<tr>
<td>Cytochrome oxidase activity, (k)-min (1)-mg protein(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without l-Arg</td>
<td>11.7 ± 0.2</td>
<td>9.2 ± 0.2*</td>
</tr>
<tr>
<td>+0.1 mM L-Arg</td>
<td>11.3 ± 0.2</td>
<td>7.5 ± 0.4††</td>
</tr>
<tr>
<td>+0.3 mM L-Arg + 1 mM AG</td>
<td>12.2 ± 0.9</td>
<td>8.5 ± 0.2</td>
</tr>
</tbody>
</table>

Data are means ± SE from 4 to 6 samples. AG; aminoguanidine l-NMMA, \(N^6\)-monomethyl-l-arginine. *\(P < 0.05\), with respect to control; †\(P < 0.05\), with respect to control + l-arginine (l-Arg) by Student’s \(t\)-test; ‡\(P < 0.05\), with respect to hypothyroid group values without l-Arg by one-way ANOVA and Dunnett’s test.
DISCUSSION

In this study, changes in thyroid state were followed by parallel variations in systemic and liver mitochondrial O$_2$ uptake that significantly correlated with serum T$_3$ levels. Variations in mitochondrial O$_2$ uptake were observed in both resting state 4 and ADP-stimulated maximal state 3 rates; a similar respiratory control ratio in all tested situations is consistent with a conservation of the coupling of oxidative phosphorylation and electron transfer in the mitochondrial membranes.

On the basis of classic mitochondrial physiology, a clear understanding of the described modulation of cellular respiration by thyroid hormones is not yet complete. In the last years, accumulated experience have clearly evidenced that NO is able to regulate mitochondrial and systemic O$_2$ uptake in different tissues and in physiological and pathological conditions (1, 6, 24, 30). Accordingly, the results presented here show 1) a clear modulatory effect of thyroid hormones on the expression and activity of rat liver mtNOS and 2) a significant correlation between mtNOS activity and systemic or mitochondrial O$_2$ uptake. The hypothyroid condition invariably was associated to increased mtNOS protein expression and activity, which were reverted by hormone replacement; moreover, administration of T$_4$ to normal rats markedly decreased mtNOS activity. In accord to the increased mtNOS activity, isolated mitochondria from the hypothyroid group had even lower O$_2$ uptake and cytochrome oxidase activity in the presence of l-Arg, the universal substrate of NOS (Table 2). Moreover, NOS inhibitor L-NMMA mostly reverted state 3 and 4 O$_2$ uptake back to the baseline values.

Likewise, these findings are clearly representative of an increased NO production vectorially directed to the mitochondrial matrix. It is noteworthy that NO modulates mitochondrial O$_2$ by reacting with cytochrome oxidase (2, 23) and that the activity of the enzyme in vivo (24) or in vitro (25) depends on NO matrix concentration. In this way, it results that the respectively measured 8% and 40% inhibition of mitochondrial O$_2$ uptake in control and hypothyroid samples treated with l-Arg (Table 2) will follow an increase of matrix steady-state NO concentration from 10 to 50–100 nM (23). Considering the kinetics of NO utilization in liver mitochondria (25) and that in the steady state, NO production and utilization are equalized, 10–100 nM NO should depend on NO production rates of 50 to 250–500 pmol·min$^{-1}$·mg protein$^{-1}$, which are in line with the measured mtNOS activity in the different studied conditions (Fig. 1). On the basis of measured mitochondrial O$_2$ uptake and considering 36 mg of mitochondrial protein/g liver and that 70% of phosphorylating reactions contribute to the resting metabolic rate (28), it emerges that the main contribution of liver metabolic rate to systemic O$_2$ uptake (basal metabolic rate) was $\sim$5–10% and remained constant in the different thyroid status. Moreover, the increased activity of skeletal muscle mtNOS in hypothyroidism (Fig.
Finally, expression of uncoupler proteins like UCP-2 could be modified by thyroid hormones, but a correlation with mitochondrial respiration has not been found in the liver yet (20).

Changes in mtNOS activity and/or expression could result from transcriptional, translational, or posttranslational effects of thyroid hormones, likely T3, on protein biosynthesis. In accord, differences in the increase of expression and activity of mtNOS in hypothyroidism (Fig. 1) suggest that thyroid-dependent changes include both transcriptional and posttranscriptional events. Further observations are required to define the structure of mtNOS, the mechanism of signaling, and the characteristics of the regulatory process in other tissues.

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