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HO-1 is located in liver mitochondria and modulates mitochondrial heme content and metabolism

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ABSTRACT **This study investigated whether inducible HO-1 is targeted to mitochondria and its putative effects on oxidative metabolism in rat liver. Western blot and immune-electron microscopy in whole purified and fractionated organelles showed basal expression of HO-1 protein in both microsomes and mitochondria (inner membrane), accompanied by a parallel HO activity. Inducers of HO-1 increased HO-1 targeting to the inner mitochondrial membrane, which also contained biliverdin reductase, supporting that both enzymes are in the same compartmentalization. Induction of mitochondrial HO-1 was associated with a decrease of mitochondrial heme content and selective reduction of protein expression of cytochrome oxidase (COX) subunit I, which is coded by the mitochondrial genome and synthesized in the mitochondria depending on heme availability; these changes resulted in decreased COX spectrum and activity. Mitochondrial HO-1 induction was also associated with down-regulation of mitochondrial-targeted NO synthase expression and activity, resulting in a reduction of NO-dependent mitochondrial oxidant yield; inhibition of HO-1 activity reverted these effects. In conclusion, we demonstrated for the first time localization of HO-1 protein in mitochondria. It is surmised that mitochondrial HO-1 has important biological roles in regulating mitochondrial heme protein turnover and in protecting against conditions such as hypoxia, neurodegenerative diseases, or sepsis, in which substantially increased mitochondrial NO and oxidant production have been implicated.—Converso, D. P., Taillé, C., Carreras, M. C., Jaitovich, A., Poderoso, J. J., Boczkowski, J. HO-1 is located in liver mitochondria and modulates mitochondrial heme content and metabolism. *FASEB J.* 20, E000–E000 (2006)**

Key Words: cytochrome c oxidase · NOS · oxygen consumption

Heme oxygenase 1 and 2 (HO-1 and HO-2) catalyze the rate-limiting step in the oxidative degradation of heme and is therefore involved in the control of cellular heme content (1–3). HO cleaves the meso-carbon bridge of the heme molecule to yield equimolar quantities of biliverdin-IX, carbon monoxide (CO), and free iron. Biliverdin-IX is subsequently converted to bilirubin-IX by NAD(P)H:biliverdin reductase (BvR), whereas the iron is sequestered in ferritin or released with the aid of iron transporter.

HO-1 has antioxidant properties attributed to bilirubin (4), which scavenges reactive oxygen species (ROS) (5), or to ferritin induction after release of the central iron from heme (6). In the same way, we recently reported that HO-1 down-regulates the expression of cytochrome *b*558 from NAD(P)H oxidase, secondary to a reduction of heme availability (7).

HO-1 is located in the endoplasmic reticulum, anchored by a single transmembrane domain at the extreme carbonyl terminus (8). HO-2 is localized in the cellular microsomal fraction (9). Recently, HO-1 was detected in caveolae from endothelial cells, where its activity is modulated by caveolin-1 (10). Although there are few references in the specialized literature (11), a potential location of HO-1 in mitochondria is important for different reasons. First, many components of the electron transfer chain (ETC) are heme-containing proteins encoded by the mitochondrial genome, whose synthesis and degradation occurs completely in the mitochondria; the heme prosthetic group ensuring protein stability and function. This is applied to subunits I-III of cytochrome oxidase (COX) (12), which is a critical determinant of O_2 uptake (13). Second, the activity of the ETC regulates not only O_2 uptake, but also results in ROS production. Indeed, 2–5% of utilized oxygen is univalently reduced to superoxide anion and to its Mn-superoxide dismutase-mediated product of dismutation, hydrogen peroxide (H_2O_2) (14). Oxygen consumption and ROS production by ETC are interdependent since binding of NO to COX reversibly inhibits O_2 uptake and increases ROS production (15). Third, recent studies provided evidence about NO synthesis in the mitochondria by a NO synthase (NOS) located in this organelle (named mitochondrial NOS, mtNOS) (16, 17). In liver, mtNOS is a posttranslational variant of nNOS-α targeted to mitochondria. Interestingly, and similarly to COX, NOS contains heme as the

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prosthetic group that determines protein stability and function (18). Considering the different activities contributing to modulation of O_2 uptake and ROS production and the increasingly recognized role of mitochondrial ROS in cellular signaling (17, 19), a putative mitochondrial location of HO-1 could be important for *in situ* control of COX and NOS expression, *via* regulation of mitochondrial heme content.

Therefore, the aim of this study was to analyze whether HO-1 is targeted toward mitochondria and to explore its effects on mitochondrial metabolism, and on COX and mtNOS expression and activity, which could be additional pathways for HO-1 antioxidant effects. We choose rat liver as a valid target, because in this organ endoplasmic HO-1 and mtNOS content are tightly regulated (17, 19).

MATERIALS AND METHODS

Experimental design

Adult female Sprague-Dawley rats (250–300 g wt) were divided in 3 groups according to intraperitoneal (i.p.) administration of: *1)* the HO-1 inductor hemin (50 mg/kg body wt); *2)* hemin plus the inhibitor of HO activity tin-protoporphyrin IX [SnPP, 35 mg/kg body wt (8)]; and *3)* both hemin and SnPP vehicles (NaOH 1M in PBS, control group). In addition, in a subset of experiments, some animals received SnPP alone and in another subset, other animals were inoculated with *E. coli* lipopolysaccharide (LPS, 2 mg/kg). Twenty-four hours after inoculation, animals were anesthetized and killed, and the liver was surgically excised. The study followed international ethical guidelines for laboratory animals and was approved by the local Institutional Animal Care and Use Committee.

Mitochondria purification

Liver mitochondria were isolated from rat by differential centrifugation in MSHE (0.22 M mannitol, 0.07 M sucrose, 0.5 mM EGTA, 2 mM HEPES/KOH, pH 7.4) supplemented with 0.1% BSA at 4°C, and purified by Percoll gradient (30% Percoll, 0.225 M mannitol, 1 mM EGTA, 25 mM HEPES/KOH, 0.1% BSA) to remove contaminating organelles and broken mitochondria, as described previously (16). The pellet (mitochondrial fraction) was subjected to three washes with MSHE plus BSA, KCl 150 mM and MSHE alone. Mitochondria were resuspended in MSHE. Microsomes and cytosol fractions (SI) were obtained by ultracentrifugation at 100,000 *g* for 30 min. Protein content was assessed by Lowry (20), and the samples were stored at −80°C.

Preparation of mitochondrial fractions

Mitochondrial fractions were obtained by hypotonic disruption and differential centrifugation, as described previously (19). Briefly, mitochondria were broken by water treatment and centrifuged at 12,000 *g* at 4°C for 10 min; supernatant represents outer membrane and intermembrane space (OM+IS) and pellet, mitoplasts (MP). Mitoplasts were further disrupted by sonication (two pulses of 40W for 10 s), and centrifuged at 8,000 *g* to discard unbroken mitoplasts; supernatant was centrifuged at 100,000 *g* for 30 min. Supernatant represents matrix fraction (M) and pellet, inner membrane fraction (IM), respectively.

Mitochondrial respiratory activities

Oxygen uptake was determined polarographically with a Clark-type electrode placed in a 3-ml chamber at 30°C, in a reaction medium consisted of 0.23 M mannitol, 70 mM sucrose, 30 mM Tris·HCl, 4 mM $MgCl_2$, 5 mM Na_2HPO_4/KH_2PO_4, and 1 mM EDTA, pH 7.4, saturated with room air (220 μM O_2) with 0.5–1 mg mitochondria protein/ml. Oxygen uptake was determined with 6 mM malate-glutamate as substrate, in the presence (state 3) or the absence (state 4) of phosphate acceptor (0.2 mM ADP). Respiratory control ratio (RCR) was calculated as state 3/state 4 respiration rate. The P/O index was calculated as the ratio of nanomoles of added ADP per nanogram of atoms of oxygen used during state 3 of respiration.

HO-1 activity

HO activity was assessed by bilirubin production in microsomal and mitochondrial pellets, using 0.5-mg proteins, as described previously (21). Bilirubin production was measured spectrophotometrically and expressed as picomoles of bilirubin per milligram of protein per hour ($\varepsilon_{453\ nm}$ = 40 $mM^{-1}\ cm^{-1}$).

NOS activity

NO production was assessed by conversion of L-[^{3}H]arginine to L- [^{3}H]citrulline, as described (22), with minor modifications (17). Specific activity was determined after subtracting the remaining activity in the presence of 10-fold excess of N^G-monomethyl-L-arginine (L-NMMA).

Mitochondrial production of hydrogen peroxide

H_2O_2 production was continuously monitored in a Hitachi F-2000 spectrofluorometer (Hitachi Ltd., Tokyo, Japan) with excitation and emission wavelengths at 315 and 425 nM, respectively, as described previously (23). To explore effects mtNOS activity on mitochondrial H_2O_2 production rate, the assay was initiated either by supplementation of 0.1 mM L-arginine or, comparatively, with 2 μM antimycin. To assess specific mtNOS-dependent-H_2O_2 production rates, 1 mM L-NMMA was added in the appropriate cases to the mitochondrial preparations. To make uniform the maximal H_2O_2 production rate, mitochondrial preparations were supplemented in all cases with 1 mM manganese superoxide dismutase (SOD)-mimetic TBAP.

Western blot analysis

Western blots were performed as described previously (23). Incubation with primary antibodies was performed overnight (1:1,000 dilution for anti nNOS isoform, monoclonal antibody (mAb); 1:5,000 dilution for anti-HO-1 and anti-HO-2, monoclonal antibodies; 1:5,000 dilution for anti-BvR, polyclonal antibody and 1:5,000 dilution for anti-COX subunits I and VIc monoclonal antibodies). Bands were detected by chemiluminescence using enhanced chemiluminescence Kit and quantified by measuring optical density (OD). Transfer efficiency was 100% in the different experiments, as verified by Coomasie blue staining in the gels before and after transfer. Because there are no uniformly expressed proteins in the different fractions, loading control and characteriza-

tion of the different samples required additional use of antibodies directed to specific markers. We used antibodies anti-VDAC-1, calreticulin, β-actin, and complex I subunit 39 kDa as markers of mitochondrial outer membrane (24), microsomes (25), cytosol (26), and mitochondrial inner membrane (27) respectively.

Enzyme activities

Cytochrome oxidase activity was determined by following the oxidation of 50 μM reduced cytochrome *c* in a Hitachi U-3000 spectrophotometer at 550 nM; ($\varepsilon_{550\ nm}$=21 mM^{-1} cm^{-1}) (28). The rate of the reaction was determined as the pseudo-first-order reaction constant, and expressed as *k* (min^{-1}) mg $protein^{-1}$. Malate dehydrogenase activity was monitored by following the oxidation of NADH at 340 nM. Extinction coefficient used for NADH was 6.22 mM^{-1} cm^{-1} (29). 5′-nucleotidase activity was determined by measuring the rate of inorganic phosphate release (30). Results were expressed in micromoles of inorganic phosphate released per minute per milligram of protein. Glucose (Glc)-6-phosphatase activity was measured by monitoring the release of phosphate from Glc-6-phosphate; 40 μg of the sample were incubated at room temperature in 200 μl HEPES (50 mM): 100 mM KCl, 2.5 mM EGTA, 2.5 mM $MgCl_2$, and 1 mM Glc-6-phosphatase; pH 7.2. The released phosphate was measured by adding 300 μl of HCl 1N, containing 10 mg/ml ammonium molybdate and 0.38 mg/ml malachite green. The standard used to measure phosphate was ATP 1 μM. After 15 min incubation, the absorbance was measured at 620 nM; the results are expressed in $nmol \cdot mg^{-1} \cdot min^{-1}$.

Immunoelectron microscopy

Immunoelectron microscopy was carried out as described previously (31). Immunohistochemistry was performed using anti-HO-1 (monoclonal) and BvR (polyclonal) antibodies (1:50). Secondary antibodies were made in goat and conjugated with colloidal gold 12-nm (antimonoclonal) and 6-nm (antipolyclonal) diameter particles. Control reactions were performed without the addition of primary antibody (Ab) and by incubation with a mixture of isotype IgG and normal rabbit serum at 1:50, as controls for HO-1 and BvR antibodies respectively. Specimens were observed in a Zeiss electron microscopy (EM)-109-Turbo transmission electron microscope at 80 kV.

Heme content measurement

Heme content was determined spectrophotometrically (390–650 nM) as described before (7, 21). Heme content was expressed as picomoles/mg proteins.

Reverse transcription PCR amplification

Total RNA of liver rats was prepared with TRIzol reagent. A PCR was performed using the Access RT-PCR System (Promega) and the following couples of primers: COX IV subunit I, Sense: 5′-ATTAAGGGCCCGTCAAGATT-3′ and antisense: 5′-GGGTTCGAATCCTTCCTTTC-3′ nNOS, Sense: 5′-GAATACCAGCCTGATCCATGGAA-3′ and antisense: 5′-TCCTCCAGGAGGGTGTCCACCGCATG-3′. DNA probes were amplified by an initial cycle at 94°C for 3 min followed by cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 1 min, and ending with a 5-min extension at 72°C. PCR products were separated on 2% agarose gels.

Absorption spectra of cytochromes

Liver mitochondria were suspended at 1 mg/ml in buffer 100 mM phosphate (pH 7.2). Spectra were differentially obtained between 500 to 650 nM wavelength in reference and sample cuvettes in the U-3000 Hitachi spectrophotometer; to detect reduced cytochromes, sodium dithionite was added to the sample cuvettes after recording the baseline. The relative amount of cytochromes was calculated by measuring the absorbances at the respective given wavelength pairs (32).

Materials

mAb antineuronal NOS (N-31020) was from Transduction Labs. (Lexington, KY). Monoclonal anti-HO-1 and HO-2 antibodies, polyclonal anti-BvR Ab, and polyclonal anticalreticulin antibodies were from StressGen (Victoria, BC, Canada). Monoclonal anti-COX subunits I and VIc antibodies and antisubunit 39 of complex I were from Molecular Probes (AA Leiden, Netherlands). Monoclonal anti VDAC1 Ab was from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies for immunogold experiments were from Jackson InmunoResearch Laboratories (West Grove, PA). Peroxidase-labeled antimouse Ab (NIF 825) was from Amersham Pharmacia. L-[2,3,-^{3}H]-arginine was from NEN (Boston, MA). SOD mimetic Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (TBAP) was from Calbiochem (San Diego, CA). Hemin chloride and SnPP-IX were purchased from Porphyrin Products (Logan, UT). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis

Data are expressed as means ± SEM; Student's *t* test, one-way ANOVA and Dunnett test were used when appropriate. Statistical significance was accepted when $P < 0.05$.

RESULTS

Mitochondrial population

Mitochondrial population was almost pure and expressed normal constituents like VDAC1, COX subunit 1, COX subunit VIc, and complex I subunit 39 and were not contaminated by other subcellular fractions and particularly by microsomes (**Figs. 1, 2,** 5 and 6). In accord, activity of microsomal markers 5′-nucleotidase and Glc-6-phosphatase in mitochondrial samples were 1% and 5%, respectively, of the original subcellular fraction (**Table 1**), and organelles did not express microsomal or cytosolic markers. In addition, activities of mitochondrial enzymes cytochrome *c* oxidase and malate dehydrogenase, were 11 and 22%, respectively, of mitochondria in microsomes, raising the possibility of minimum microsomal contamination by mitochondrial fraction (Table 1). However, these low activities were partly nonspecific because in the different experiments, microsomal protein blotting did not reveal specific mitochondrial markers like VDAC1, COX subunit VIc, and complex I subunit 39 (Figs. 1, 5 and 6).

Additional evidence of purity and homogeneity of the obtained mitochondrial fraction was given by EM studies (Fig. 1*C*). Moreover, the organelles were func-

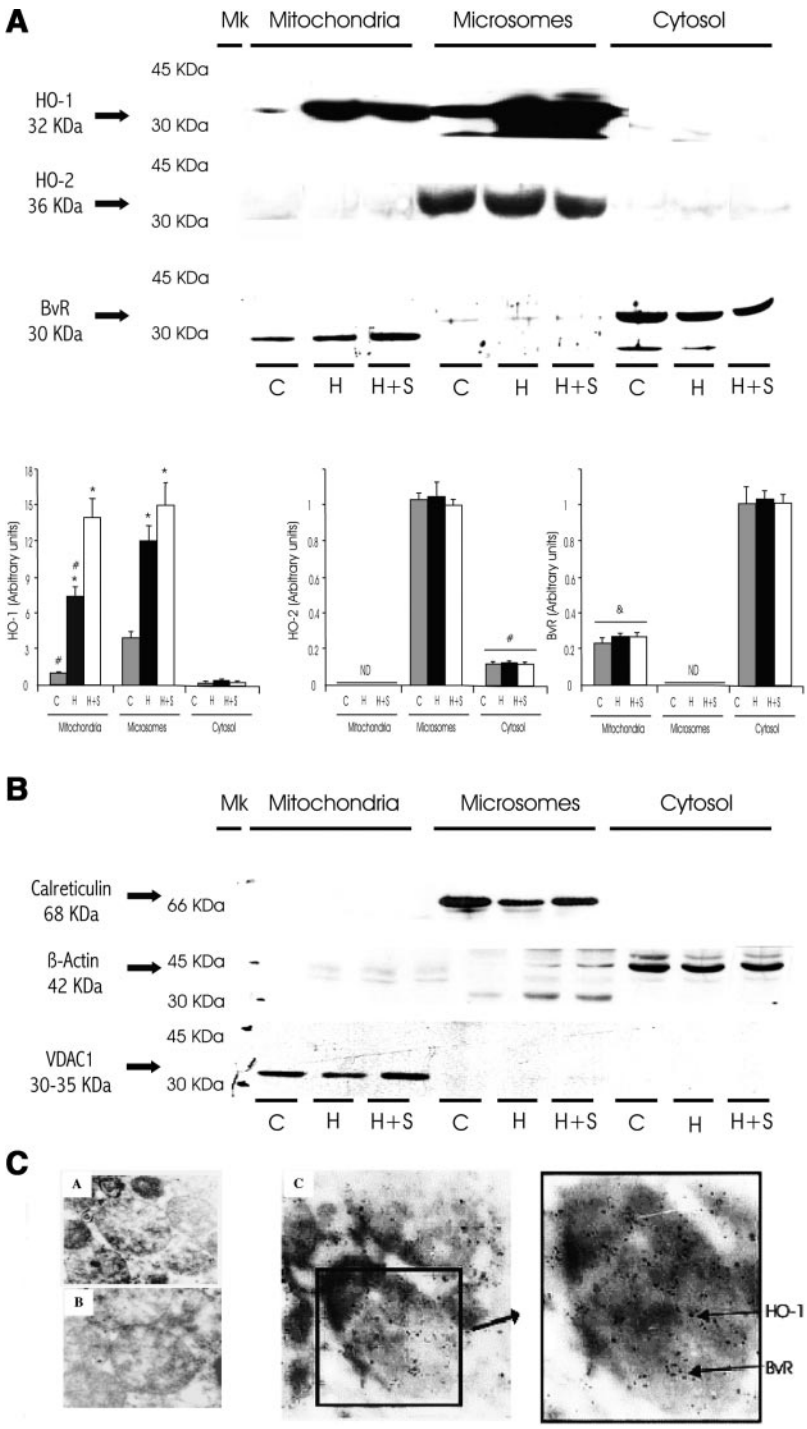

Figure 1. HO and biliverdin reductase (BvR) expression in microsomes and mitochondria. *A)* Typical Western blot analysis of HO-1, HO-2, and BvR expression in a control (C) animal and in animals treated with hemin (H) and hemin plus tin-protoporphyrin-IX (H+S) in mitochondria, microsomes, and cytosol. Densitometric analysis of proteins expression is shown in the lower part of the panel. Each bar represents the mean ± SEM OD of positive signal (n=4). *Different from the respective control organelle, $P < 0.05$; #Different from the microsomes. &Different from the cytosol. *B)* Typical Western blot analysis of calreticulin (microsomal marker), β-actin (cytosolic marker), and VDAC1 (mitochondrial marker) in all fractions from control and treated animals. These Western blots are representative of four different experiments. Densitometric analysis did not show differences between the different conditions in each fraction (data not shown). *C)* Immunoelectronic microscopy analysis of HO-1 and BvR expression in mitochondria isolated from a control animal. HO-1 and BvR were detected with secondary antibodies conjugated with 12 nM and 6 nM colloidal gold, respectively, (*C*). Control experiments performed by omitting the primary antibodies (*A*), and by replacing them with a mixture of a isotype IgG corresponding to the anti-HO-1 Ab plus nonimmune rabbit serum (*B*).

tionally active with acceptable respiratory control indexes (**Table 2**).

HO-1 but not HO-2 is constitutively expressed in rat liver mitochondria

We attempted first to detect HO-1 in mitochondria. As expected, Western blot analysis revealed canonical constitutive HO-1 in rat liver microsomes, but also expression of HO-1 in mitochondria isolated from the control group (Fig. 1*A*). In contrast with HO-1, HO-2 was solely expressed in microsomes (Fig. 1*A*).

HO-1 colocalizes with BvR in mitochondria

Because enzyme conversion of biliverdin to bilirubin is a key component of the HO system (33), we further examined the expression of BvR in mitochondria. Western blot experiments showed that BvR was expressed in the mitochondria of control rats as well (Fig. 1*A*). As described by Maines and coworkers (34), BvR migrated as two bands with molecular masses of 33 and 30 kDa, respectively. In accord with these authors, the 33 kDa band was predominant in cytosol. The band in mitochondria migrated predom-

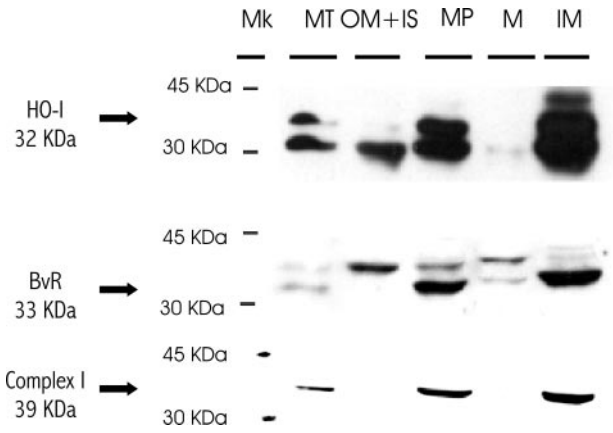

Figure 2. Localization of HO-1 and BvR in control mitochondria. Mitochondria were subfractionated by lysis and ultracentrifugation as described in the Materials and Methods section. Western blot shows the fine localization of both HO-1 and BvR in mitoplasts and inner mitochondrial membrane; the inner membrane was recognized by using an Ab anti 39-kDa subunit of complex I. MT, mitochondria; OM, external membrane; IS, intermembrane space; MP, mitoplasts; M, mitochondrial matrix; and IM, inner membrane.

inantly at 30 kDa, reflecting probably posttranslational modifications, as previously reported (35).

To confirm HO-1 and BvR colocalization by another method, we practiced immune EM with double marking with colloidal gold. The experiments confirmed the presence of HO-1 and BvR in mitochondria (Fig. 1*C*); almost all small or large gold particles (5–10 nM) were inside mitochondria, and the procedure was specific since no particle could be detected in the mitochondria in the absence of anti-HO-1 and -BvR antibodies, and by replacing them with a mix of isotype IgG and rabbit serum.

Next, we discerned about submitochondrial localization of HO-1. Western analysis of isolated submitochondrial fractions showed that HO-1 was mainly localized in the inner mitochondrial membrane (Fig. 2). BvR was also localized in the mitochondrial inner membrane (Fig. 2). This localization provides a steric advantage for direct HO effects on the respiratory components.

Modulation of mitochondrial HO-1 expression and activity

Having demonstrated a constitutive protein expression and activity of HO-1 in liver mitochondria, we evaluated whether these phenomena were modulable by different HO-1 inducers. Twenty-four hours after administration, the classic HO-1 inductor hemin (8), increased HO-1 expression in liver microsomes and also protein targeting to mitochondria (Fig. 1 *A*). Interestingly, in comparison to the control situation, hemin induced a higher HO-1 expression in mitochondria than in microsomes (7 vs. 3.5 fold, $P < 0.05$, Fig. 1*A*).

As expected, HO activity was detected in microsomes (**Fig. 3*B***). Mitochondrial HO-1 expression was accompanied by a detectable HO activity, measured *ex vivo* in isolated organelles. This activity represented ~15% of that of the microsomal fraction (Fig. 3*A*, $P<0.05$). Hemin increased microsomal and mitochondrial HO activities by near 50% and 25%, respectively. The effect of hemin was abolished by administration of HO-1 inhibitor SnPP, confirming the efficacy of hemin as a HO-1 inducer in our model (Fig. 3*A* and *B*). SnPP alone decreased significantly HO activity (Fig. 3*A* and *B*). Increase of mitochondrial HO activity was observed as well 24 h after LPS inoculation (Fig. 3*A*). These results suggest that *1)* in the absence of a challenge, a basal HO-1 activity is detectable in mitochondria; and *2)* after stimulation, HO-1 is targeted to mitochondria, associated with a critical cytosol concentration, and irrespective of the utilized enzyme inducer.

In contrast to HO-1, neither microsomal HO-2 nor mitochondrial or cytosolic BvR expression were modified by hemin administration (Fig. 1*A*).

Modulation of mitochondrial heme content

Considering HO-1 variations, we first examined putative effects on the mitochondrial heme content. Animals treated with hemin exhibited a significant reduction in mitochondrial heme content ($P<0.05$), which was significantly reversed by the concomitant administration of SnPP ($P<0.05$, **Fig. 4**). Furthermore, hemin + SnPP-treated animals exhibited a four-fold increase in mitochondrial heme content as compared to control animals

TABLE 1. *Contamination indexes*

	Mitochondria	Microsome	% Contamination
Cytocrome *c* oxidase (*K*/min/mg proteins)	19 ± 3	2 ± 0.5	11
Malate dehydrogenase (nmol/min/mg proteins)	890 ± 24	198 ± 26	22
5′-Nucleotidase (μmol/min/mg proteins)	0.1 ± 0.09	10 ± 1	1
Glucose-6-phosphatase (nmol/min/mg proteins)	0.3 ± 0.01	5.7 ± 0.04	5.3

Data are means ± SE from 6 independent experiments. Cytochrome *c* oxidase and malate dehydrogenase are mitochondrial markers, and 5′ nucleotidase and glucose-6-phosphatase are microsomal markers.

TABLE 2. *Mitochondrial oxygen uptake*

	Control	Hemin	SnPP
Mitochondrial O_2 uptake			
ngat O_2/min/mg protein			
State 4			
Without L-Arg	30 ± 5	26 ± 5	25 ± 5
+ 0.3 mM L-Arg	26 ± 1	21 ± 1	30 ± 1
+ 0.3 mM L-Arg + 3 mM NMMA	24 ± 1	24 ± 1	25 ± 1
State 3			
Without L-Arg	126 ± 6	167 ± 6*	136 ± 4
+ 0.3 mM L-Arg	103 ± 2	141 ± 1	114 ± 1
+ 0.3 mM L-Arg + 3 mM NMMA	124 ± 2	145 ± 1	148 ± 1
RCR			
Without L-Arg	4.74 ± 1.10	6.44 ± 0.39*	4.79 ± 0.64
+ 0.3 mM L-Arg	3.96 ± 0.70	5.83 ± 0.24	3.80 ± 1.34
+ 0.3 mM L-Arg + 3 mM NMMA	5.17 ± 0.24	6.59 ± 0.74	5.49 ± 1.42

Data are means ± SE from 6 independent experiments. L-NMMA, NG-monomethyl-L-arginine. Respiratory control rate (RCR) is defined as the state 3 / state 4 respiration rate. *Different from Control animals, $P < 0.05$.

($P<0.05$). In contrast with these data, no significant change in heme content was observed in microsomes in hemin and hemin plus SnPP animals (Fig. 4).

Modulation of COX expression and activity by HO-1

Having demonstrated that HO-1 induction in mitochondria was associated with modulation of mitochondrial heme content, we examined the association between HO-1 modulation and expression and activity of COX, the heme-containing enzyme that regulates oxygen consumption by ETC.

COX activity was significantly reduced in hemin-treated rats as compared to control animals ($P<0.05$, **Fig. 5*A***); this effect was reversed by coadministration of SnPP ($P<0.05$).

To search structural bases for decreased activity, we followed the visible spectrum of COX. In accord to Vanneste (32), a 20% decrease in peak of cyt *a*+*a3* at 605 nM was observed after hemin treatment. This decrease is consistent with a reduction of content from 0.45 ± 0.02 to 0.35 ± 0.02 nmoles per milligram of mitochondrial protein, respectively (Fig. 5*B*), likely due to high HO-1 concentration in the organelles. No significant changes were detected in the other mitochondrial cytochromes.

To investigate if decreased COX spectrum observed in hemin-treated animals was related to a decreased expression of protein subunits, we performed Western

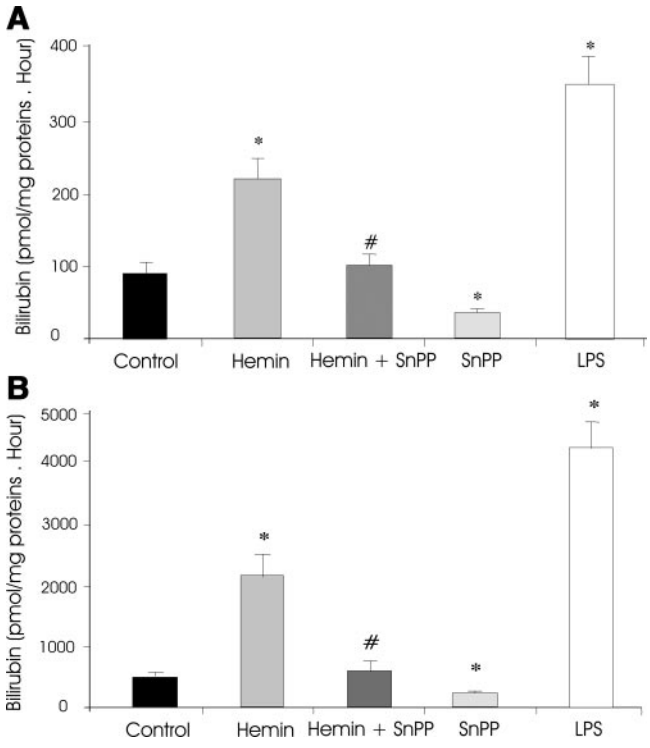

Figure 3. HO activity. Liver HO activity was quantified by evaluating spectrophotometrically the production of bilirubin at 340 nm in mitochondria (*A*) and microsomes (*B*, 0.5 mg protein in each case) in same conditions than in Fig. 1, and in animals treated with SnPP alone. For comparative purposes, we also measured HO activity after injection of mice with 10 μg *E. coli* lipopolysaccharide (LPS) i.p. Each bar represents mean ± SEM (n=5). *$P < 0.05$ vs. control, #$P < 0.05$ vs. hemin.

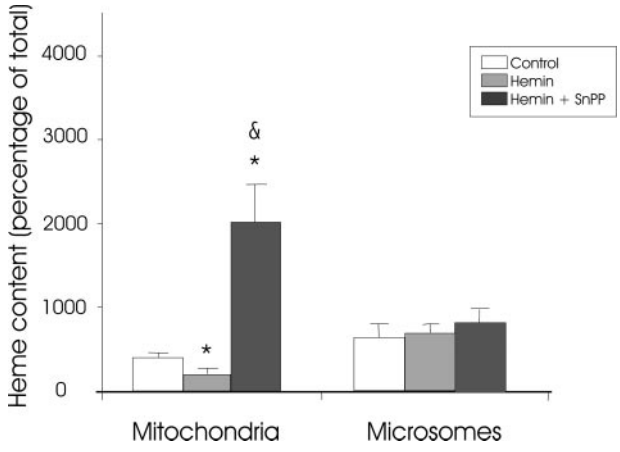

Figure 4. Heme content in the subcellular fractions. Each bar represents the mean ± SEM (n=5). *Different from control animals, $P < 0.05$; &Different from hemin-treated animals, $P < 0.05$.

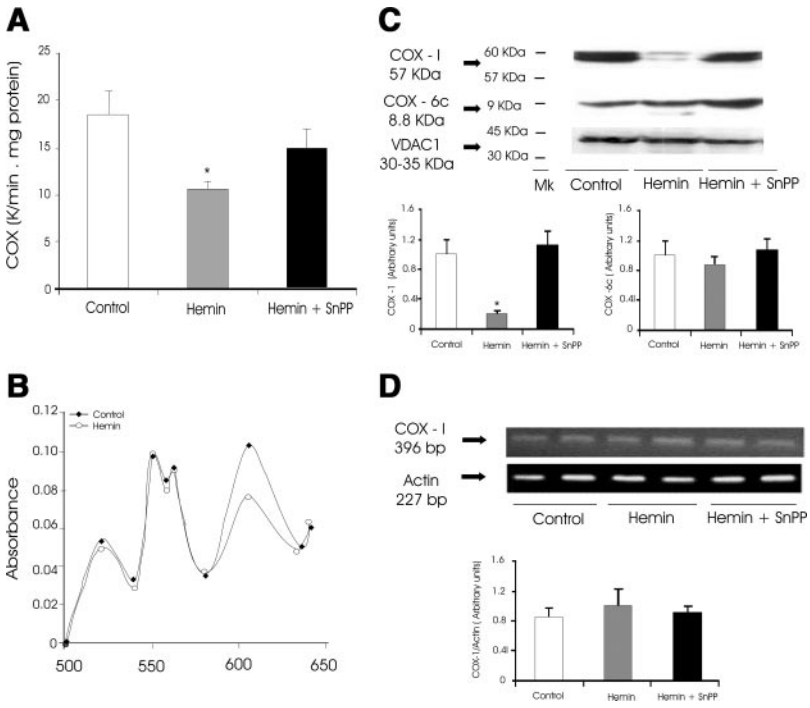

Figure 5. Expression and activity of cytochrome *c* oxidase (COX) follows HO-1 activity in mitochondria. *A)* COX activity in the different groups of animals. Each bar represents the mean ± SEM (n=4). *Different from control, $P < 0.05$. *B)* Spectrum analysis of COX showing at 605 nM, corresponding to cyt $a+a3$, after hemin treatment. *C) Top*: Typical Western blot analysis of COX subunits I and VIc and VDAC1 expression in mitochondria from a control (C) animal and from animals treated with hemin and hemin plus SnPP. The lower part of the panel shows densitometric analysis of COX subunits I and VIc expression. Each bar represents the mean ± SEM OD (n=4). *Different from control, $P < 0.05$. *D)* RT-PCR analysis of COX subunit I mRNA expression in 2 animals from the different groups. Abbreviations are the same as in *C*.

blots using antibodies raised against 2 COX subunits, which are coded by the mitochondrial and the nuclear genome, and which bind and not bind heme (subunits I and VIc respectively; (13)). The results of these experiments showed a selective decreased expression of subunit I in hemin-treated animals, whereas expression of subunit VIc was unchanged, irrespective of the treatment (Fig. 5*C*). SnPP alone did not modify the expression of COX subunit I (data not shown), but prevented the effect of hemin, when both compounds were administered simultaneously (Fig. 5*C*).

Because transcription of mRNA expression of COX subunits could be regulated by heme content (13), we next analyzed mRNA expression of subunit I, and found that it remains unchanged, irrespective of the treatment used (Fig. 5*D*).

Modulation of mitochondrial NOS expression and activity by HO-1

Having demonstrated that HO-1 modulated COX activity, we next investigated the effects of HO-1 on mtNOS, the other major modulator of mitochondrial oxygen uptake and ROS production. As described previously, mtNOS expression was detected using an Ab directed against nNOS-α (17). Therefore, mtNOS expression and activity were significantly lower in hemin-treated as compared to control rats ($P<0.05$, respectively), and this effect was prevented by SnPP (**Fig. 6*A*** and ***B***). The microsomal expression of nNOS in control adult rats was low and was not significantly modified in hemin-treated animals as compared to controls (Fig. 6*B*). Expression of mRNA from nNOS was not modified by protoporphyrins administration (Fig. 6*C*).

Modulation of mitochondrial oxygen consumption and ROS production by mitochondrial HO-1

Because HO-1 modulated COX and mtNOS expression and activity, we investigated its effects on mitochondrial oxygen consumption and H_2O_2 production.

As compared to control animals, mitochondria isolated from hemin-treated rats showed no significant modification of state 4 respiration rate, but a significant increase in state 3 respiration rate (25%, $P<0.05$, Table 2). Accordingly, the RCR was significantly higher in hemin-treated as compared to control rats (6.44±0.39 vs. 4.74±1.10, $P<0.05$). SnPP alone did not modify states 4 and 3 respiration rates (data not shown) but prevented the effect of hemin, when both compounds were administered simultaneously (Table 2).

We wondered whether the increase of state 3 respiration rate observed in hemin-administered animals was related to a relative lack of mitochondrial NO, secondary to the decreased expression and activity of mtNOS. Incubation of isolated mitochondria with L-arginine (300 μM) produced a similar decrease of state 3 O_2 uptake rate in both control and hemin-treated animals (Table 2). However, addition of L-NMMA reverted this phenomenon, only in control rats. Indeed, the contribution of NO to the state 3 respiration was 17% in control animals and only 3% in hemin-treated animals ($P<0.05$, Table 2). The effect of hemin was prevented by SnPP (Table 2). These data are consistent with an effect of HO-1 on the respiratory chain via the reduction in mtNOS activity.

Because NO modulates H_2O_2 production by the mitochondria (36), we investigated NO-dependent and independent H_2O_2 production in the different groups of animals. Mitochondria from hemin-treated animals

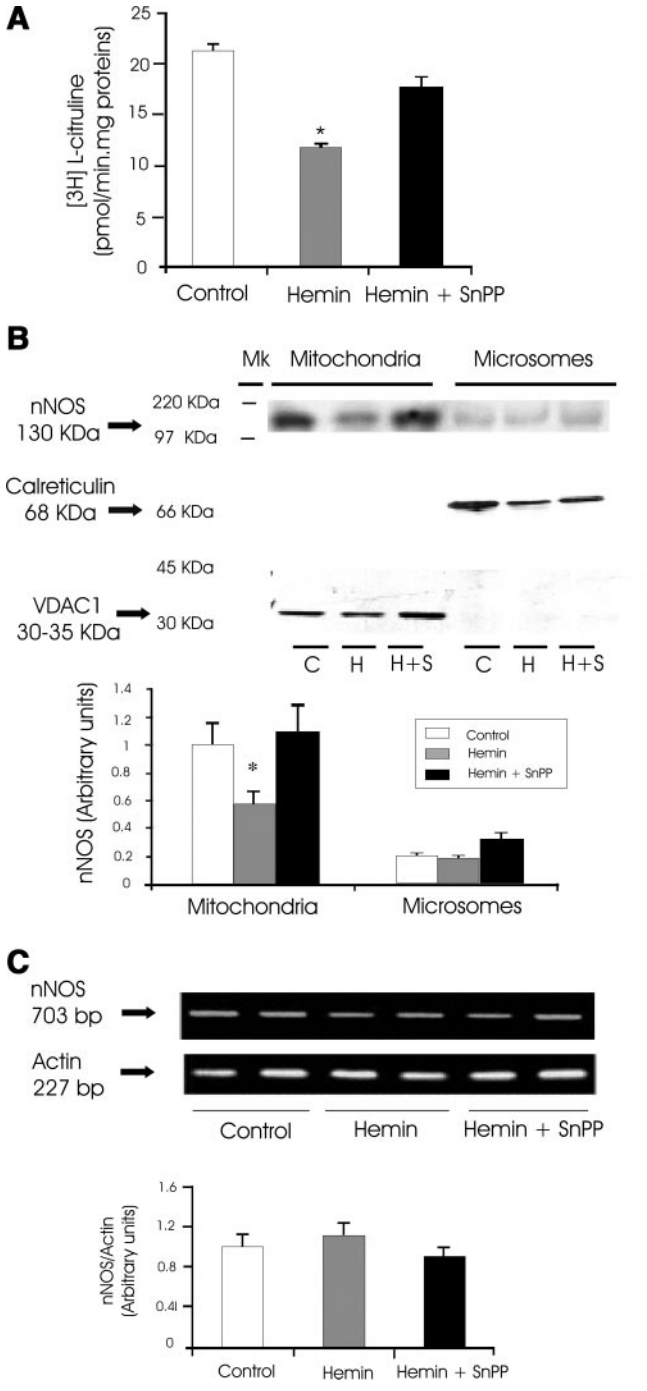

Figure 6. Mitochondrial NOS expression and activity depends on HO-1. *A)* Mitochondrial NOS activity in the different groups of animals. Each bar represents the mean ± SEM (n=5). *Different from control, $P < 0.05$. *B)* Typical Western blot analysis of neuronal NOS, calreticulin, and VDAC1 expression in mitochondria and microsomes from a control (C) animal and from animals treated with hemin and hemin plus SnPP (H and H+S respectively). The lower part of the panel show densitometric analysis of neuronal NOS expression. Each bar represents the mean ± SEM OD (n=4). *Different from control, $P < 0.05$. *C)* RT-PCR analysis of neuronal NOS mRNA expression in the 2 animals from the different groups. Abbreviations are the same as in Fig. 5*C*.

supplemented with antimycin showed a similar production rate of H_2O_2 than organelles from control animals, reflecting a similar maximal capability to produce nondiffusible superoxide radicals, the products of the univalent reduction of oxygen (**Fig. 7*A***). In the presence of L-arginine, H_2O_2 production was significantly lower in mitochondria from hemin-treated rats than in controls ($P<0.05$, Fig. 7 *B*), reflecting the lower activity of mtNOS. In fact, the effect of L-arginine was ascribed to mtNOS, as it was inhibited by 1 mM NOS inhibitor L-NMMA (data not shown).

DISCUSSION

In this study, we show that, in addition to microsomes, HO-1 protein is located in liver mitochondria, operates on redox components through heme availability and, as a part of its antioxidant properties, modulates mitochondrial O_2 uptake and ROS production.

Mitochondrial HO activity has been reported in liver of rodent species infected with *Plasmodium berghei* or treated with the HO-1 inductor cobalt chloride (11) but was absent in *ox* heart mitochondria (37), a discrepancy probably explained on the basis of species and/or organ differences. Furthermore, heme degradation ac-

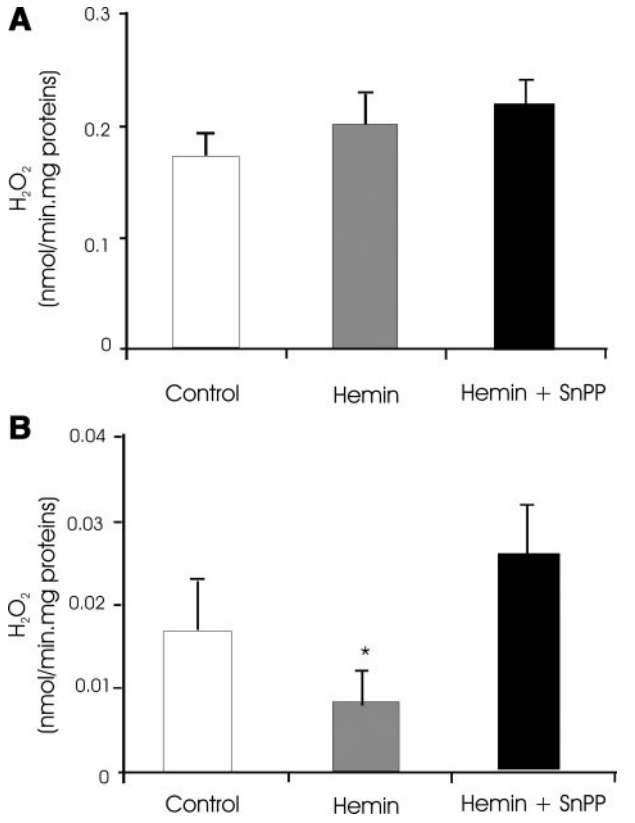

Figure 7. Mitochondrial H_2O_2 yield. *A)* Mitochondrial H_2O_2 production in the presence of antimycin (maximal production). Each bar represents the mean ± SEM (n=5). *B)* Mitochondrial NOS-dependent H_2O_2 production in the different groups of animals. Each bar represents the mean ± SEM (n=5). *Different from control, $P < 0.05$.

tivities other than HO-1 have been found in mitochondria, either associated with H_2O_2 production by complex I (37) or with purified lipoamine dehydrogenase activity (38). Although we cannot exclude the existence of these pathways in rat liver mitochondria, the concomitant expression of BvR and HO-1 found in the present study and previous data on the existence of mitochondrial ferritin with ferroxidase activity (39) indicate that all components of the HO system can be expressed in these organelles.

The modulation of mitochondrial HO-1 by hemin or LPS closely followed the variations in the microsomal fraction. In purified mitochondria with negligible microsomal contamination, mitochondrial HO-1 activity and content were in ~10–20% of that of microsomes. This percentage indicates that only a fraction of induced HO-1 is targeted to mitochondria suggesting that, tightly controlled HO-1 import could be limited by the intrinsic translocon activity (40). HO-1 lacks N-*termini* targeting presequence to mitochondria and thus, as transporters colocalized in the inner membrane, it probably requires internal hydrophobic targeting domains, and a specific import machinery, like the Tim 23 transporter (41). It must be noted that in controls, the ratio of HO-1 activity to protein expression was close in mitochondria and microsomes (330 and 450, respectively, Figs. 1*A* and 3). After hemin treatment, there was an apparent reduction of this ratio in mitochondria compared to microsomes (53 vs. 275, respectively) that could depend on a significant degradation of mitochondrial heme proteins, which are an important fraction of total mitochondrial proteins, leading to an overestimation of HO-1 expression.

HO-1 targeting to mitochondria was associated with significant variations of mitochondrial heme pool. In fact, in hemin-treated animals, a different subcellular distribution of total heme content (0.82×10^{-6} M, similar to reported by Ren and Correia (42)) exclusively resulted from 50% decrease of mitochondrial heme, without variations in microsomes. Heme redistribution could be the product of an imbalance between degradation and synthesis, both mitochondrial processes. Although at 5–100 μM (43), heme itself inhibits gene transcription and mitochondrial import of ALA synthetase (44) and coproporphyrinogen oxidase (45), coadministration of hemin and HO-1 inhibitor SnPP increased mitochondrial heme content by 10-fold with respect to hemin alone, thus supporting a main role of mitochondrial HO-1 in the regulation of mitochondrial heme pool.

The reduction of mitochondrial heme content resulted in a decreased expression and activity of COX, the terminal acceptor of the ETC. This finding did not depend on variations of the number of mitochondria because at the same liver weight, decrease of COX from hemin-treated animals was selectively associated with a decreased protein expression of heme-bound subunit I, whereas expression of heme-independent subunit, VIc remained unchanged. Because in hemin-treated animals, mARN expression of subunit I was not modified, the effect of HO-1 is likely to be posttranslational and secondary to the decreased mitochondrial heme content. In accord, all processing of subunit I occurs in the mitochondria: subunit I is coded on mitochondrial DNA, translated on mitochondrial ribosomes, and heme incorporation takes place locally (13). Concerning the order for progression of heme catabolism and cytochrome degradation, it was reported many years ago that prior heme depletion subsequently decreases complex IV (46). It is difficult to ascertain here the mutual relationship between heme and protein synthesis or degradation. Heme is incorporated in the process of folding of subunit I within the membrane, even promoting binding of subunit II, which, in turn, could stabilize the binuclear heme a3/CuB center of subunit I (12). A critical role of heme incorporation on subunit I expression and COX stability and function was demonstrated in human fibroblasts where heme deficiency secondary to inhibition of ferrochelatase decreased protein expression of subunit I and COX activity (46), whereas independency of heme pool and protein expression of subunit VIc was reported in the mouse liver (47). According to references and experimental findings, we surmise that *1*) HO-1 activity impedes proper COX assembly by decreasing mitochondrial heme content and likewise, *2*) heme-depleted free COX subunit I is prone to being degraded in the mitochondrial matrix.

Similarly to COX subunit I, HO-1 could affect mtNOS content because nNOS protein dimerization and stability depend on heme availability (18), and consequently, the heme-deficient monomeric form of nNOS is selectively ubiquitinilated and degraded (48), even in mitochondria (49). Because in hemin-treated animals, liver nNOS mRNA expression and a small amount of microsomal nNOS did not change, the *in situ* effect of targeted HO-1 on mtNOS protein is remarkable. Considering that CO produced by HO-1 activity binds to heme and inhibits electron transfer (50), some contribution of CO to COX or mtNOS inhibition remains to be elucidated. It is worth notice that COX turnover is lower than that of NOS, suggesting that, at the same mitochondrial HO-1 activity, NOS should be more decreased than COX in the presence of hemin. However, it is quite difficult to discern whether a proportional correlation between heme availability and NOS/COX activities exists, as they depend on other facts like dimerization (NOS monomerization is required for degradation), molar proportion between heme and the respective proteins, and activities of the degrading enzymes. Moreover, although the different types of heme in the two proteins (common heme in NOS or exclusively mature heme-*a* in COX) are equally catabolized, differential effects of these substrates on HO activity cannot be ruled out.

Oxygen uptake and the weight of electron transfer rate would be balanced through the relative participation of [NO] and $[O_2]$, as evidenced by differential HO-1 effects on the turnover of mitochondrial-targeted NOS and COX. It is noteworthy that despite 40%

decreased COX activity, we found no modification in state 4 oxygen uptake rate, and even a significant increase of state 3 oxygen uptake rate was observed in hemin-treated animals. This effect could be due to a threshold effect, as the relative COX capacity is in excess with respect to that required to support the endogenous respiration rate (51). Alternatively, HO-1 modulates other heme proteins involved in mitochondrial metabolism. A concomitantly decreased mtNOS content and activity helps to explain the observed variations of O_2 uptake in hemin-treated animals. NO produced by mitochondria is vectorially directed to the matrix and reversibly inhibits cytochrome oxidase by binding to heme (nitrosyl-Fe^{2+}) (36). Likewise, hemin-induced HO-1 targeting was associated with decreased mtNOS and thus, O_2 uptake increased and was less sensitive to NOS substrate L-arginine (Table 2).

In addition to the effects on oxygen utilization, mitochondrial NO promotes a high production rate of superoxide anions and of the dismutation product, hydrogen peroxide (36). This effect relies on inhibition of electron transfer at transition between cytochromes *b* and *c1* and on direct reactions between ubiquinol and NO, leading to ubisemiquinone formation that transfers electrons to O_2 to form superoxide anion. Accordingly, mitochondria from hemin-treated animals with low mtNOS had 50% less NO-dependent H_2O_2 yield than controls. This effect could contribute to the antioxidant properties of HO-1, especially in conditions associated with increased mtNOS expression, such as endotoxemia (52). Furthermore, a decrease in mitochondrial NO production could facilitate heme degradation by HO-1, as endogenous NO attenuates heme degradation by HO, secondary to heme nitrosylation (53).

Mitochondrial HO-1 localization and the regulation of NOS and COX provide a new insight in the functions and biological implications of HO-1 and, more broadly, in the mechanisms controlling the mitochondrial metabolism as *in situ* modulator of respiratory hemoproteins. For instance, HO-1 is rapidly induced after a single hypoxic event (54) or by hypoxia-inducible factor (55), which could indicate its role in the adjustment of mitochondrial O_2 requirements. As a sensor of O_2 and also NO yields (56), HO-1 could participate in cell signaling and life and death programs. By the same reasons and by the reduction in NO-dependent ROS production, mitochondrial localization could explain protective properties of HO-1 in conditions such as neurodegenerative diseases (57), ischemia reperfusion (58) or sepsis (59), in which substantially increased mitochondrial NO and ROS production has been implicated (52, 60). FJ

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