Effect of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes

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STADLER, JOSEF, TIMOTHY R. BILLIAR, RONALD D. CURRAN, DENNIS J. STUEHR, JUAN B. OCHOA, AND RICHARD L. SIMMONS. *Effect of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes.* Am. J. Physiol. 260 (Cell Physiol. 29): C910–C916, 1991.—Although nitric oxide (•N=O) biosynthesis is inducible in rat hepatocytes (HC), the physiological significance of •N=O production by these cells is unknown. Short exposure of HC to authentic •N=O led to a concentration-dependent inhibition of mitochondrial aconitase, NADH-ubiquinone oxidoreductase, and succinate-ubiquinone oxidoreductase (complexes I and II of the mitochondrial electron transport chain). Most susceptible to •N=O inhibition was mitochondrial aconitase, in which a reduction in enzyme activity to 20.2 ± 1.6% of control was observed. In contrast to mitochondrial aconitase, cytosolic aconitase activity was not inhibited by •N=O. After exposure to a maximal inhibitory concentration of •N=O, mitochondrial aconitase activity recovered completely within 6 h. Complex I did not fully recover within this incubation period. Endogenous •N=O biosynthesis was induced in HC by a specific combination of cytokines and lipopolysaccharide. After 18 h of incubation with these stimuli, a significant inhibition of mitochondrial aconitase activity to 70.8 ± 2.4% of controls was detected. However, this was due only in part to the action of •N=O. A non-•N=O-dependent inhibition of mitochondrial function appeared to be mediated by tumor necrosis factor.

aconitase; reduced nicotinamide adenine dinucleotide-ubiquinone oxidoreductase; succinate-ubiquinone oxidoreductase; 4Fe-4S cluster; tumor necrosis factor-α

AN UNSTABLE INTERMEDIATE with the chemical and pharmacological profile of nitric oxide (•N=O) has been identified in mammalian nitrogen metabolism (14, 15, 19, 22). This short-lived radical is generated from the oxidation of one of the two equivalent guanidino nitrogens of L-arginine by a •N=O synthase, leaving L-citrulline. •N=O is unstable in aerated solutions and decomposes to nitrite and nitrate (14, 26). •N=O biosynthesis has been reported in a variety of different cell types including endothelial cells (15, 22), macrophages (14, 19), neutrophils (25), and cerebellar neurons (10). We have shown that Kupffer cells (KC), like other macrophages (27), produce •N=O in response to endotoxin (3). Furthermore, we have demonstrated that hepatocytes (HC) synthesize significant quantities of •N=O when exposed to supernatants generated by endotoxin-activated KC (5). Signals for in vitro induction of HC •N=O production have been identified as interferon-γ, tumor necrosis factor-α, interleukin-1, and lipopolysaccharide (6). Inducible cytosolic •N=O synthase activity in HC has also been demonstrated following in vivo injection of *Corynebacterium parvum* (2).

On the basis of the diversity of cell types shown to produce •N=O, a wide range of biological functions is likely to exist; however, on the intracellular level only a few actions have been reported. These effects can be divided into activation or inhibition of specific enzymes. Soluble guanylate cyclase is activated by •N=O, leading to an increase in guanosine 3′,5′-cyclic monophosphate (cGMP) and the induction of biological responses that are dependent on cGMP as a second messenger (29). In murine tumor cell lines, three mitochondrial enzymes are inhibited by macrophage-derived •N=O (8, 9, 11). These include mitochondrial aconitase (tricarboxylic acid cycle), NADH-ubiquinone oxidoreductase, and succinate-ubiquinone oxidoreductase (complex I and complex II of the mitochondrial respiratory chain). Direct inhibition of complexes I and II has been demonstrated in murine tumor cells by exposure to authentic •N=O (14, 28). •N=O-induced inhibition appears to be the result of an interaction with 4Fe-4S clusters, which are prosthetic groups of these enzymes (9, 23).

In HC, the induction of endogenous •N=O synthesis as well as the exposure to authentic •N=O is accompanied by a significant decrease in total protein synthesis on a posttranscriptional basis (6a). Whether these changes are due to impaired energy production is not known. In addition, there is evidence that alterations in HC mitochondrial function are associated with liver dysfunction in sepsis (4). Because •N=O production is induced in HC by inflammatory stimuli, it was of interest to investigate the effects of authentic and cell-generated •N=O on HC mitochondrial function.

EXPERIMENTAL PROCEDURES

Reagents. Nitrogen gas (N_2; oxygen-free grade) and authentic nitric oxide gas (•N=O) were obtained from Linde Specialty Gases (Danbury, CT). Williams medium E, minimal essential medium (without L-leucine), L-glutamine, L-arginine HCl, penicillin, streptomycin, EDTA-trypsin, and dialyzed calf serum were purchased from GIBCO Laboratories (Grand Island, NY). Bovine insulin was from Eli Lilly (Indianapolis, IN), heat-inac-

tivated low-endotoxin calf serum was from Hyclone Laboratories (Logan, UT), and L-[4,5-^{3}H(N)]leucine was from New England Nuclear (Boston, MA). Recombinant murine tumor necrosis factor-α (rmTNF) was obtained from Genzyme (Cambridge, MA), recombinant human interleukin-1-β (rhIL-1) was from Cistron Technology (Pine Brook, NJ), recombinant rat interferon-γ (rrIFNg) was from Amgen Biologicals (Thousand Oaks, CA), and lipopolysaccharide from *E. coli* 0111:B_4 (LPS) was from Difco Laboratories (Detroit, MI). N^G-monomethyl-L-arginine acetate was prepared as previously described (3). All other reagents were purchased from Sigma Chemical (St. Louis, MO).

HC isolation. Liver cells were harvested from unfasted male Sprague-Dawley rats (200–300 g, Harlan Sprague-Dawley, Madison, WI) using a collagenase perfusion technique (6). After injection of 600 U heparin sodium, the portal vein was cannulated and the liver initially perfused in situ with 250 ml calcium-free solution containing 0.145 M NaCl, 6.7 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and 2.4 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). The liver was then excised, and a second perfusion containing 0.05% collagenase (type IV), 67 mM NaCl, 6.7 mM KCl, 100 mM HEPES, and 1% bovine serum albumin was carried out on an ex vivo recirculating apparatus for 20 min. Flow rate was 40 ml/min, and the perfusates were kept at 37°C. The parenchyma was then gently combed to produce a cell suspension, and the HC were purified by repeated differential sedimentations at 50 *g* for 2 min. Purity was >96%, and viability exceeded 85% as determined by trypan blue exclusion.

Exposure to •N=O. •N=O-saturated medium, [•N=O] $\approx$ 1.25 mM (16, 28), was produced by deoxygenating serum-free medium with N_2 gas for 30 min and then bubbling authentic •N=O gas into the medium for 20 min. •N=O gas was first passed over KOH pellets to remove nitrogen dioxide. The fully saturated solution was then diluted with deoxygenated medium to the appropriate concentrations. HC were suspended in these solutions at a concentration of 1×10^6 cells/ml in gastight tubes that had been flushed with N_2. Control medium was prepared in the same manner, except that •N=O was volatilized by gassing the medium with N_2 for 30 min for a second time. After 2 min of exposure, the cells were centrifuged for 3 min at 50 *g* and resuspended in oxygenated respiration medium. All steps were carried out at 4°C.

Cell culture technique. All culture incubations were carried out at 37°C and 95% O_2-5% CO_2. Culture medium consisted of Williams medium E supplemented with 15 mM HEPES, 2 mM L-glutamine, 10^5 U/l penicillin, 100 mg/l streptomycin, 10^{-6} M insulin, 10^{-8} M dexamethasone, and 10% calf serum. For determination of radiolabeled leucine incorporation, freshly harvested HC were plated at 2×10^5 HC/ml on gelatin-coated 96-well tissue culture trays in 0.1 ml/well. For measurements of mitochondrial respiration, HC were incubated at 1×10^6 HC/ml in 75-cm^2 flasks in 12 ml/flask. To harvest the cells, the flasks were gently washed with serum-free medium and reincubated for 5 min with 6 ml of 0.05% EDTA-trypsin to detach the cells. The trypsin was inactivated by addition of 6 ml medium containing 10% calf serum.

Induction of endogenous •N=O synthesis. We have recently shown that a combination of three cytokines and LPS induce •N=O production by HC to a similar extent as was observed with conditioned KC supernatant (6). This combination includes rmTNF (500 U/ml), rhIL-1 (5 U/ml), rrIFNg (100 U/ml), and LPS (10 μg/ml). These agents were added to HC in culture medium as described above, and the cells and supernatants were harvested after 18 h.

Respiration measurements. Respiration medium contained 250 mM sucrose, 2.0 mM H_2KPO_4, 10 mM HEPES, 0.5 mM EGTA, and 0.7% bovine serum albumin (BSA). The respiration medium and all substrate solutions were titrated to pH 7.2. The plasma membrane was made permeable to the substrates by exposure to 0.0075% digitonin for 1 min followed by three washes at 400 *g* at 4°C (11). Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH). After 5 min of equilibration at 37°C, state 3 and state 4 respiration were determined as described (21). Citrate, isocitrate, or malate was added as either indirect or direct NAD-linked substrates (final concentration 5 mM each). 2-Hydroxymalonate and L-glutamate served as exchange partners for substrate transport systems of the inner mitochondrial membrane (5 mM each) (9). To measure oxygen consumption mediated by complex II (5 mM succinate) or by donation of electrons to coenzyme Q (10 mM α-glycerophosphate), electron flow from complex I to the mitochondrial electron transport chain was inhibited by rotenone (100 nM). To determine oxygen consumption by selective electron donation to cytochrome *c*, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) was used as an artificial electron donor (TMPD 0.2 mM/ascorbate 1 mM) after inhibition of electron flow from complex III to cytochrome *c* with antimycin A. Complex IV activity was inhibited by addition of KCN (3 mM) and corrected for cyanide-insensitive oxygen consumption ($\approx$15%). The activity of the other complexes was totally blocked by KCN. TMPD and KCN solutions were made fresh daily. The electrode signal was fed into a strip-chart recorder that was calibrated over a 0–100% scale. Oxygen consumption was calculated assuming an initial air-saturated [O_2] = 390 ng/ml (11). Respiratory activities mediated by complexes I and II involve the sequential activities of complexes III and IV within the mitochondrial electron transport chain. Likewise, complex III involves complex IV activity. Because oxygen consumption was not limited by one of the following steps, we refer only to the activity of the enzyme complex where the electron transport was started.

Aconitase activity assay. Direct measurement of aconitase activity was based on the disappearance of *cis*-aconitate detected spectrophotometrically at 240 nm (13). To determine total aconitase activity (cytosolic and mitochondrial), HC were permeabilized with digitonin and the disappearance of *cis*-aconitate measured in the presence of 2-hydroxymalonate as an exchange partner for the mitochondrial transporter. By washing the cells after permeabilization, all substrates for the mitochon-

drial transporter and most of the cytosolic aconitase activity were removed. The remaining cytosolic enzyme activity was taken to be the amount of activity detected in the absence of 2-hydroxymalonate. This was subtracted from the amount of activity in the presence of 2-hydroxymalonate to obtain mitochondrial activity. Complete cytosolic aconitase activity was then calculated by subtracting mitochondrial from total activity. The assays were performed in respiration medium in the presence of 0.02% BSA at 37°C to obtain enzyme activities comparable to those obtained from oxygen consumption measurements. The reaction was started with the addition of 0.2 mM *cis*-aconitate and stopped by the addition of 50 μl/ml Dowex 50.

Determination of protein synthesis. HC total protein synthesis was measured using a 4-h labeling interval with [^{3}H]leucine (5.0 Ci/mmol, 1.0 μCi/well) added to minimal essential medium without L-leucine. The label was terminated by lysing the HC with 1 N NaOH with 0.025% Triton X-100. The protein was precipitated with 40% trichloroacetic acid and collected onto filter paper using a multichannel cell harvester. [^{3}H]leucine incorporation was determined by liquid scintillation counting.

Nitrite/nitrate assay. The stable end products of L-arginine-dependent •N=O production, nitrite (NO_2) and nitrate (NO_3), were measured using an automated procedure based on the Griess reaction (12).

Statistical analysis. Results are expressed as means ± SE. Where indicated, significance between groups was determined using the Student's unpaired *t* test.

RESULTS

•N=O inhibition of specific mitochondrial enzymes of HC. Aconitase catalyzes the interconversion of citrate and isocitrate with *cis*-aconitate as an intermediate. To determine the effect of •N=O on mitochondrial aconitase of HC enzyme activity, aconitase-mediated state 3 respiration was measured by the addition of citrate to the respiration medium. After the conversion to isocitrate, NADH is formed by NAD^+-isocitrate dehydrogenase and other dehydrogenases of the tricarboxylic acid cycle, which serves as a substrate for complex I of the mitochondrial electron transport chain. A second direct assay of aconitase activity was performed to ensure that citrate-dependent oxygen consumption after exposure to •N=O reflected aconitase activity and not merely changes in the activity of isocitrate dehydrogenase or one of the components of the mitochondrial electron transport chain. In this assay of enzyme activity, the disappearance of *cis*-aconitate was the indicator for aconitase activity. In both assays, 2-hydroxymalonate was used as an exchange partner for the tricarboxylic acid carrier of the inner mitochondrial membrane. As demonstrated in Fig. 1, exposure of HC to •N=O solutions for 5 min led to a concentration-dependent impairment of the activity of mitochondrial aconitase. Citrate-dependent oxidation rates were decreased to 70.8 ± 6.2% of control HC by a 5% solution, to 27.1 ± 1.6% by a 10% solution, and to 20.2 ± 1.6% of control by a 20% solution. The degree of inhibition was nearly identical to the direct assay of the disappearance of *cis*-aconitate.

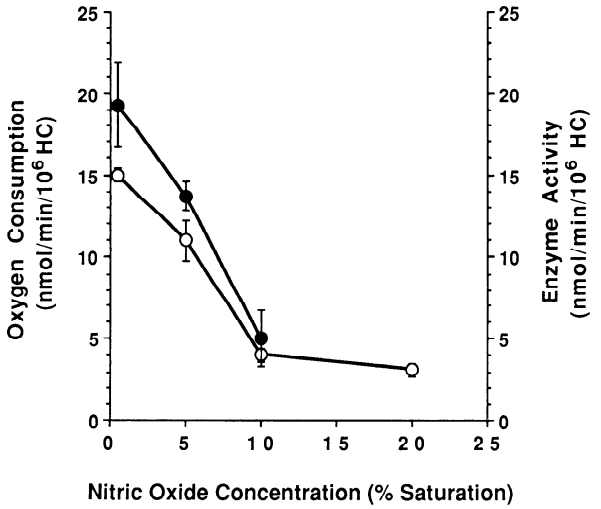

FIG. 1. Concentration-dependent inhibition of rat hepatocyte (HC) mitochondrial aconitase activity of HC by authentic nitric oxide (•N=O). Freshly harvested HC were exposed to solutions with different •N=O saturation for 5 min. Medium was then changed to respiration medium and cells permeabilized by addition of digitonin. After 3 washes, mitochondrial aconitase activity was measured as citrate-dependent state 3 respiration (○) or with an enzymatic assay based on the disappearance of *cis*-aconitate (●). Values represent means ± SE of 3 or 4 experiments.

TABLE 1. *•N=O inhibits mitochondrial but not cytosolic aconitase activity*

	Aconitase Activity, nmol·min^{-1}·10^6 HC^{-1}		
	Total	Cytosolic	Mitochondrial
Fresh HC	72.0±1.1	51.0±4.1	22.5±3.8
Control HC	69.3±5.6	50.0±7.0	19.3±2.6
5% •N=O	64.7±5.2	51.0±5.8	13.7±0.9
10% •N=O	53.2±10.7	48.3±9.3	5.0±1.7

Values are means ± SE of 3 experiments. Total aconitase activity and mitochondrial aconitase activity were differentiated by using the disappearance of *cis*-aconitate as an indicator for the enzyme activity before and after washing of permeabilized rat hepatocytes (HC). Difference was calculated as cytosolic activity.

By measuring total and mitochondrial enzyme activity with the direct enzyme assay, cytosolic aconitase activity was calculated as the difference between the two values. Approximately two-thirds of the total activity was attributable to the cytosolic enzyme, and the remaining one-third was measured in the mitochondrial fraction (Table 1). A •N=O-induced decrease of cytosolic aconitase activity was not detectable, while the decrease in mitochondrial enzyme activity accounted for all of the decrease measured in total activity.

Measuring the activity of the complexes of the mitochondrial electron transport chain, we found that both NADH-ubiquinone oxidoreductase (complex I) and succinate-ubiquinone oxidoreductase (complex II) were inhibited by exogenous •N=O. As illustrated in Fig. 2, exposure to •N=O solutions induced a concentration-dependent inhibition of state 3 respiration of both enzymes. State 4 respiration was not influenced (results not shown). At all concentrations, complex I was more sensitive to •N=O inhibition than complex II. After exposure to the maximal •N=O concentration tested

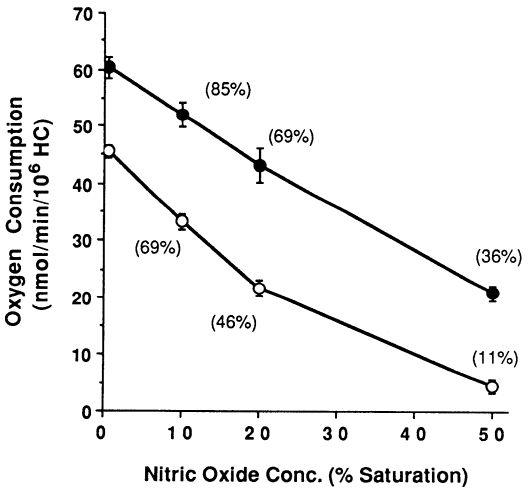

FIG. 2. Concentration-dependent inhibition of NADH-ubiquinone oxidoreductase (complex I) (○) and succinate-ubiquinone oxidoreductase (complex II) (●) of the mitochondrial electron transport chain. After exposure of HC to solutions with increasing •N=O concentrations for 5 min, medium was changed to respiration medium and HC permeabilized by addition of digitonin. After 3 washes, complex I activity was measured as oxygen consumption with malate/glutamate as the specific substrate and with succinate as substrate for complex II. Enzyme activity percent of control HC is given in parentheses. Results represent means ± SE of 3 experiments.

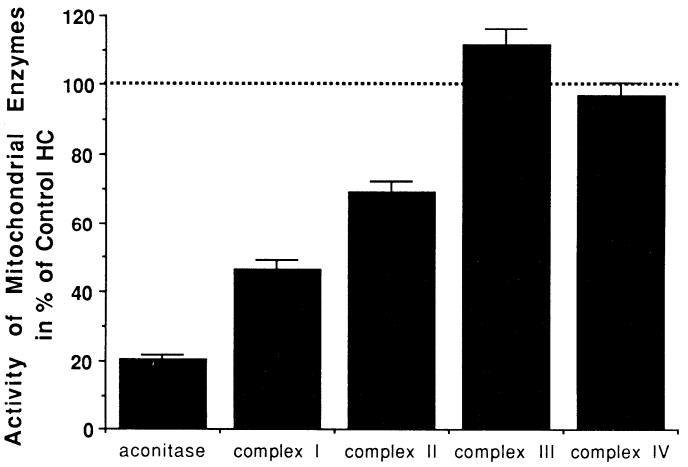

FIG. 3. Differences in susceptibility of mitochondrial enzymes to •N=O inhibition. HC were exposed to 20% •N=O-saturated solutions for 5 min, permeated, and then enzyme activities determined as substrate-dependent oxygen consumption (aconitase, citrate/hydroxymalonate; complex I, malate/glutamate; complex II, succinate; complex III, α-glycerophosphate; complex IV, TMPD/ascorbate). Results are expressed as relative activity compared with HC which were exposed to control medium. Values represent means ± SE of 3–5 experiments.

(50%), activity of complex I was decreased to 10.9 ± 0.8% of controls, while the activity of complex II was 35.9 ± 1.8% of control HC. Both enzyme complexes were less susceptible to •N=O inhibition than mitochondrial aconitase. Differences in sensitivity of mitochondrial aconitase and of the four complexes of the mitochondrial electron transport chain to •N=O inhibition are shown in Fig. 3. Compared with control HC, exposure to a 20% •N=O solution for 5 min reduced mitochondrial aconitase activity to 20.2 ± 1.6%, complex I to 46.3 ± 3.0%, and complex II to 69.2 ± 3.1%. In contrast, oxygen consumption mediated by complex III (via α-glycerophosphate dehydrogenase), and complex IV-mediated respiration rates were not significantly affected.

Recovery of HC from •N=O injury. To study the kinetics and the extent of recovery from •N=O inhibition following exposure to 20% •N=O solution for 5 min, the medium was changed to oxygenated culture medium thereafter and the HC were incubated at 37°C for 90 min, 3 h, and 6 h. Citrate was added as a substrate for mitochondrial aconitase and isocitrate for complex I of the mitochondrial electron transport chain. As shown in Fig. 4, mitochondrial aconitase activity was decreased to 22.0 ± 1.8% of control cells at the beginning of the incubation period, but within 90 min increased to 58.4 ± 3.1% of control cells and then to 90.4 ± 2.9% after 3 h. By 6 h, a complete recovery of aconitase activity was found. Although complex I-mediated respiration was inhibited to only 55.0 ± 4.7% of control immediately after •N=O exposure, at 6 h recovery was still incomplete, reaching only 86.3 ± 3.3% of controls.

Deoxygenation, NO_2^-/NO_3^- contamination, and alkalosis of •N=O solutions did not affect respiratory activity. After deoxygenation, oxygen concentration in the medium was lower than detectable with the Clark-type oxygen electrode (<2 nM dissolved O_2/ml). In addition, the saturation of the solution with •N=O led to a contamination with 1.98 ± 0.09 mM NO_2^-/NO_3^- (n = 4). While deoxygenation increased the pH from 7.4 to 8.5, the contamination with NO_2^-/NO_3^- induced by exposure to •N=O decreased the pH back to 8.0. Thereafter, during the short exposure to •N=O solutions, HC were also briefly exposed to anoxia, NO_2^-/NO_3^-, and alkalosis. These three conditions had no detectable influence on state 3 mitochondrial respiration rates when compared with freshly harvested cells (Table 2). These findings suggest that the observation obtained using •N=O solutions were indeed attributable to the action of •N=O.

Endogenously produced •N=O has discrete inhibitory effects. •N=O production was induced in cultured HC to

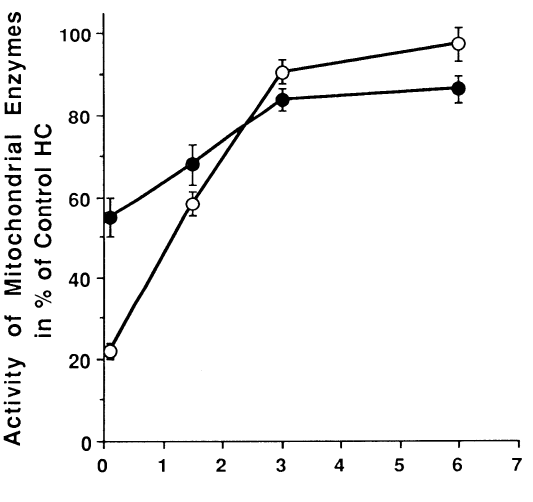

FIG. 4. Kinetics of recovery of HC from •N=O injury. After exposure to a solution of 20% •N=O saturation for 15 min, respiration rates were measured at various time intervals. Aconitase activity (○) was determined as citrate-dependent and complex I activity (●) as isocitrate-dependent oxygen consumption. Results are expressed as relative activity compared with control HC. Values are means ± SE of 3–5 experiments.

TABLE 2. *Brief exposure to anoxia, NO_2^-/NO_3^-, and alkalosis had no influence on mitochondrial respiration rates of rat hepatocytes*

Substrate	Acceptor Sites		Oxygen Consumption of Permeabilized Hepatocytes, nmol $O_2 \cdot min^{-1} \cdot 10^6$ cells^{-1}	
	TCA cycle	METC	Unaltered medium	Test medium
Citrate	Aconitase	Complex I	14.0±0.4	13.6±0.3
Isocitrate	IDH	Complex I	34.7±1.1	35.2±1.4
Malate	MDH	Complex I	45.5±2.3	45.0±2.0
Succinate	SDH	Complex II	58.4±1.6	58.3±1.4
Glycerophosphate		Coenzyme Q (via GPDH)	5.6±0.5	5.9±0.4
TMPD/ascorbate		Cytochrome *c*	66.0±2.9	67.8±3.2

Values are means ± SE of 4 experiments. Permeabilization and measurement of mitochondrial respiration were performed either immediately after HC harvesting or after exposure to test medium for 5 min. Test medium was deoxygenated, fully saturated with •N=O, and finally depleted of •N=O by passing N_2 through the solution. TCA cycle, tricarboxylic acid cycle; METC, mitochondrial electron transport chain; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; SDH, succinate dehydrogenase; GPDH, glycerophosphate dehydrogenase; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

TABLE 3. *Combination of LPS, IFNg, IL-1, and TNF induces NO_2^-/NO_3^- production and suppression of total protein synthesis of HC*

HC Culture Conditions		NO_2^-/NO_3^-, μM	Protein Synthesis
Cytokine + LPS combination	NMA (0.5 mM)		[^{3}H]leucine incorporation, counts/min
−	−	10.6±1.8	41,548±2,600
−	+	7.1±1.4	40,979±3,203
+	−	194.7±22.0*	29,899±2,843*
+	+	8.6±1.4	37,308±3,785

Values are means ± SE of 3 or 4 experiments. Freshly harvested HC were incubated for 18 h under the various culture conditions listed above. NO_2^-/NO_3^- levels were determined in culture supernatants. Cytokine combination consisted of 100 U/ml recombinant rat interferon-γ (rrIFNg), 5 U/ml recombinant human interleukin-1-β (rhIL-1), and 500 U/ml recombinant murine tumor necrosis factor-α (rmTNF), also including 10 μg/ml lipopolysaccharide (LPS). NMA, N^G-monomethyl-L-arginine. * $P < 0.001$ vs. regular medium.

determine whether endogenous •N=O exerts a similar inhibitory effect on mitochondrial respiration as exogenous •N=O. A specific combination of cytokines and LPS is known to stimulate •N=O synthesis by cultured HC. After exposure to rrIFNg, rhIL-1, rmTNF, and LPS, the concentration of NO_2^-/NO_3^- in culture supernatants increased from 10.6 ± 1.8 to 194.7 ± 22.0 μM over 18 h (Table 3). Consistent with our previous reports (6), this increase in nitrogen oxide production was associated with a decrease in total protein synthesis ([^{3}H]leucine incorporation) to 71.5 ± 2.9% of controls. Both HC NO_2^-/NO_3^- formation and the inhibition of protein synthesis were effectively prevented by the addition of 0.5 mM N^G-monomethyl-L-arginine, an inhibitor of •N=O biosynthesis (5, 14). Figure 5 illustrates that exposure to cytokines and LPS also induced a significant inhibition of mitochondrial aconitase activity to 70.8 ± 2.4% of controls. This effect was partially reversed by N^G-monomethyl-L-arginine (88.0 ± 3.0% of controls). Complexes I and II were inhibited to a lesser extent with almost no effect of N^G-monomethyl-L-arginine. Complex IV activity was only slightly decreased with exposure to cytokines and LPS.

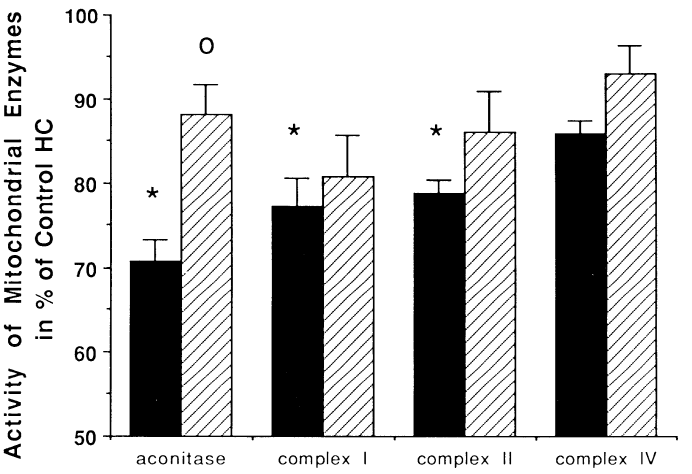

FIG. 5. Effect of endogenously produced •N=O on activity of specific mitochondrial enzymes. HC were incubated with LPS and a combination of cytokines, consisting of rrIFNg, rhIL-1, and rmTNF to induce L-arginine-dependent •N=O generation (■). N^G-monomethyl-L-arginine was added (▨) to prevent •N=O formation. After 18 h, substrate-dependent oxygen consumption was measured to determine mitochondrial enzyme activity (aconitase, citrate; complex I, isocitrate; complex II, succinate; complex IV, TMPD/ascorbate). Results are expressed as relative activity compared with control HC cultured in standard medium with and without N^G-monomethyl-L-arginine. Values represent means ± SE of 3 experiments. See text for definitions. * $P < 0.01$ vs. controls. ° $P < 0.003$ vs. cytokine combinations.

TABLE 4. *TNF inhibits mitochondrial respiration independent from •N=O generation*

Culture Conditions	Oxygen Consumption, nmol·min^{-1}·10^6 cells^{-1}		Protein Synthesis	NO_2^-/NO_3^-, μM
	Citrate	Isocitrate	[^{3}H]leucine incorporation, counts/min	
Standard medium	8.7±0.2	22.7±0.7	41,507±2,529	6.6±3.1
+ 10 μg/ml LPS	9.2±0.4	22.7±0.6	44,939±3,717	3.7±1.0
+ 100 U/ml rrIFNg	8.6±0.4	23.4±0.7	41,033±3,271	5.2±1.4
+ 5 U/ml rhIL-1	8.7±0.5	24.6±1.3	45,871±3,932	10.8±2.5
+ 500 U/ml rmTNF	6.3±0.3*	16.6±0.6*	49,274±1,954	8.5±1.7

Values are means ± SE of 3 or 4 experiments. Freshly harvested HC were incubated for 18 h under listed conditions. All media contained 1 mM L-arginine. •N=O levels were determined in the 18-h supernatant. Aconitase complex I activity is expressed as citrate- or isocitrate-dependent oxygen consumption. Definitions are as in Table 3. * $P < 0.001$ vs. standard medium.

TNF inhibits mitochondrial respiratory activity independent from •N=O. Because the inhibition of •N=O synthesis failed to reverse all of the suppression of mitochondrial enzyme activity, the effect of each of the agents used for induction of endogenous •N=O production was tested. No single substance increased HC NO_2^-/NO_3^- production or decreased protein synthesis (Table 4). A significant inhibition of citrate- and isocitrate-dependent oxygen consumption was induced by TNF, whereas no effect was seen with LPS, IFNg, or IL-1 alone.

DISCUSSION

To determine the susceptibility of HC mitochondrial enzymes to the inhibitory effect of •N=O, we exposed rat HC to authentic •N=O and measured mitochondrial aconitase, NADH-ubiquinone oxidoreductase, and succinate-ubiquinone oxidoreductase activity, all of which are known to be inhibited by authentic or cell-generated •N=O in other cell types (8, 11, 28). All three enzymes were markedly inhibited in HC in a concentration-dependent manner following a brief exposure to exogenous •N=O. Complex III and complex IV of the mitochondrial electron transport chain were not influenced by •N=O, showing that impairment of mitochondrial enzyme activity was selective. By far the most susceptible enzyme was mitochondrial aconitase. Artifacts resulting from the preparation of the •N=O solutions, namely deoxygenation, NO_2^-/NO_3^- contamination, and alkalosis, had no effect on the enzymes tested, indicating that the inhibition of enzyme activity was attributable to the action of •N=O.

HC differ from the other cell types used to study the action of •N=O in many aspects. One of these differences is a high content of cytosolic aconitase in addition to mitochondrial aconitase. We found a ratio of cytosolic to mitochondrial aconitase activity of 2.5 to 1, similar to that reported in other studies (7). Even though mitochondrial aconitase is very sensitive to •N=O inhibition, no effect of •N=O on the cytosolic aconitase was demonstrated. Whether this discrepancy is due to structural differences between the cytosolic and mitochondrial enzymes is not known, since the amino acid sequence and the ultrastructure of the two enzymes are not known. However, 4S-4Fe clusters have been demonstrated in both beef heart mitochondrial and beef liver cytosolic aconitase. A fact that may account for this observation is that the cytosolic enzyme is active in a soluble form, while the mitochondrial is part of an enzyme complex bound to the inner mitochondrial membrane (24). The lipophilic nature of •N=O may therefore explain the susceptibility of the mitochondrial enzyme to •N=O.

After exposure to a 20% •N=O-saturated solution, the activity of mitochondrial aconitase showed a complete recovery within 6 h, while the inhibition of complex I activity was not fully reversible in this time period. This shows that although HC mitochondria are rather sensitive to •N=O, the injury is short lived, and repair mechanisms exist. The mechanism of the reversal is not known but may involve the reconstitution of iron in the Fe-S cluster (17), as suggested for tumor cell aconitase (8).

A specific combination of cytokines and LPS-induced L-arginine-dependent nitrogen oxide production in rat HC. Exposure to cytokines and LPS also resulted in a moderate decrease of mitochondrial aconitase activity, while complexes I and II were inhibited to a lesser degree. Inhibition of •N=O synthesis by N^G-monomethyl-L-arginine reversed most of the aconitase inhibition, but only a small fraction of the reduction of complexes I and complex II activity, indicating that cell-generated •N=O significantly influences only mitochondrial aconitase. The relative minor effect of cell-generated •N=O compared with authentic •N=O may be due to low amounts of •N=O present at any one time during endogenous production or to the capacity of HC to overcome the ongoing •N=O inhibition by active repair mechanisms. Another explanation could be the presence or induction of a •N=O scavenger system that protects from the inhibitory effects of •N=O. In any case, we want to stress the fact that the resistance to major inhibition by endogenous •N=O is a unique feature of HC, since in tumor cells (1, 28) and macrophages (9) much smaller amounts of cell-generated •N=O lead to a near-complete inhibition of these enzymes. This circumstance may allow HC to depress mitochondrial function of other liver cells, e.g., KC or endothelial cells, by release of •N=O without affecting their own energy metabolism.

Of the three single cytokines and LPS, TNF induced a significant reduction of mitochondrial aconitase, complex I, and complex II activity without inducing •N=O biosynthesis. This shows that TNF impairs mitochondrial function through a •N=O-independent mechanism. Similar findings for TNF have been described in tumor cells (18) and are thought to be correlated to synthesis of reactive oxygen intermediates (30). An inhibitory effect of LPS on mitochondrial respiration as reported by others (20) was not observed.

We have recently demonstrated (6a) that exposure of HC to authentic •N=O inhibits their protein synthesis for up to 12 h. In the present study, most of the mitochondrial enzyme activity recovers within 1–2 h. This finding and the very small degree of mitochondrial inhibition induced by endogenous •N=O suggest that impaired mitochondrial function does not account for the decrease in protein synthesis.

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