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Metabolic Changes Induced by Cold Stress in Rat Liver Mitochondria

Concepción Bravo,[1] Martín Vargas-Suárez,[2] Sara Rodríguez-Enríquez,[1] Herminia Loza-Tavera,[2] and Rafael Moreno-Sánchez[1,3]

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The mechanisms involved in the metabolic changes induced by cold stress in isolated rat liver mitochondria were studied. Respiration, ATP synthesis, and membrane potential as well as the contents of several metabolites were determined in liver mitochondria from cold-exposed rats. At different times of cold exposure, the force–flux relationships showed net variation in flux (enhanced respiration, diminished ATP synthesis) with no associated variation in force (H^+ gradient); this suggested that decoupling rather than classical uncoupling was involved in the effects of cold stress. The flux control coefficient of the H^+ leak on basal respiration was slightly increased by 380 h of cold exposure. Cold stress also induced a diminution in *total* membrane fatty acids, Zn^{2+}, Fe^{3+}, ATP, and ADP/O ratios; the content of cytochromes $c + c_1$ and b oscillated. The contents of Ca^{2+}, Na^+, P_i, and cytochromes $a + a_3$ were not affected, whereas matrix ADP, AMP, K^+, and Mg^{2+} were markedly increased. Basal and oleic acid-stimulated respiration of mitochondria from cold-stressed rats was inhibited by GDP, carboxyatractyloside, or albumin. These agents did not affect basal respiration in control mitochondria. Western blot analysis showed enhanced expression of a protein of about 35 kDa, presumably the uncoupling protein 2, induced by long-term cold exposure. The overall data suggest that cold stress promoted decoupling of oxidative phosphorylation, and hence, changes in several matrix metabolites, by increasing *free* fatty acids and the UCP2 content.

KEY WORDS: Uncoupling protein 2; decoupling; free fatty acids; ATP synthesis; respiration.

INTRODUCTION

During hibernation and in the awakening of the ground squirrel, the mitochondrial levels of free fatty acids (FFA), and the activity of the respiratory chain in mitochondria from several tissues increase (Brustovestsky *et al.*, 1990, 1992, 1993); this brings about generation of heat. In mitochondria, this phenomenon is accompanied by lower ADP/O ratios and H^+ gradients and, in consequence, uncoupling of oxidative phosphorylation. In skeletal muscle mitochondria, isolated from cold-exposed seal (Grav and Blix, 1979), pigeon, and mouse (Skulachev, 1991), the stimulated rate of respiration and the decreased P/O ratio are restored by addition of albumin, suggesting the presence of uncoupling levels of FFA in these mitochondria.

The uncoupling of oxidative phosphorylation by FFA involves, apparently, three steps: the diffusion of protonated fatty acids across the mitochondrial inner membrane, the subsequent dissociation of H^+ into the matrix milieu, and the return of the anionic fatty acid to the external medium by a transport reaction catalyzed by the adenine nucleotide translocator (Brustovestsky *et al.*, 1990, 1992, 1993). Accordingly, the FFA-mediated activation of the respiratory chain is inhibited by carboxyatractyloside (CAT), a specific inhibitor of the adenine nucleotide translocator, and albumin (Brustovestsky *et al.*, 1990, 1992, 1993; Schonfeld, 1990).

[1] Departamento de Bioquímica, Instituto Nacional de Cardiología, México, D.F. 14080.
[2] Departamento de Bioquímica, Facultad de Química, UNAM. México, D.F. 04510.
[3] To whom all correspondence should be mailed. email: morenosanchez @hotmail.com

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On the other hand, in the mitochondria from brown adipose tissue (BAT), one of the major sites of thermogenesis in mammals, significant amounts of uncoupling protein 1 (UCP1) have been detected. This protein has a high protonophoric activity, which is inhibited by GDP and activated by FFA (for reviews see Nicholls and Locke, 1984; Ricquier and Bouillaud, 2000). In consequence, BAT mitochondria exhibit high respiratory rates and negligible ATP synthesis, even in the absence of FFA. The addition of GDP restores the ability of these mitochondria to drive energy-dependent reactions, such as oxidative phosphorylation (Nicholls and Locke, 1984) and Ca^{2+} uptake (Chávez *et al.*, 1996) at rates comparable to those of mitochondria from other tissues. Several other UCPs have been detected in other tissues, but the factors regulating their activity and expression are not well known (Ricquier and Bouillaud, 2000).

In rats, starvation, fat-rich diet, and extensive exercise lead to significant elevation in oxygen uptake, and diminution of cell ATP, ATP/ADP ratio, and the mitochondrial pH gradient in liver (Seitz *et al.*, 1977; Soboll and Stucki, 1985; Bode *et al.*, 1990). The liver of these experimental animals also exhibits high levels of FFA and long-chain acyl-CoA (Seiz *et al.*, 1977; Bakarat *et al.*, 1982; Bode *et al.*, 1990). Addition of fatty acids to perfused livers mimics the changes induced by fasting, fat-rich diet and exercise (Scholz *et al.*, 1984; Soboll *et al.*, 1984). Liver mitochondria, isolated from animals submitted to starvation or physical exercise, show respiratory control (i.e., enhanced basal respiratory rates) and ADP/O ratios lower than those of mitochondria isolated from control animals. The metabolic alterations are reversed by treatment of mitochondria with serum albumin (Klug *et al.*, 1984).

In marked contrast to the above-mentioned studies, it has been reported that cold stress does not perturb the coupling of oxidative phosphorylation in rat liver mitochondria (Aithal and Ramasarma, 1971; Mak *et al.*, 1983). This conclusion was based on the determination of the ADP/O ratio using only one oxidizable substrate, and no measurements of ATP synthesis and membrane potential were carried out. Therefore, in this work the effect of cold exposure on respiration, ATP synthesis, and membrane potential in rat liver mitochondria was studied using different oxidizable substrates. The membrane lipid composition and the content of uncoupling protein 2 (UCP2), ions and adenine nucleotides were also determined. The purpose of these studies is to gain further information on the biochemical mechanisms of adaptation to cold in liver mitochondria.

MATERIALS AND METHODS

Animals

Female Wistar rats (200–250 g) were housed at 25°C with free access to food and water. Rats under cold treatment were maintained at 4°C for the times described in each experiment with free access to food and water.

Mitochondria Preparation, Oxygen Uptake, Membrane Potential, and ATP Synthesis

Coupled rat liver mitochondria (Moreno-Sánchez, 1985) were obtained after the indicated times of cold exposure as previously described. O_2 uptake using a Clark-type O_2 electrode (Moreno-Sánchez, 1985), incorporation of $^{32}P_i$ into ATP (De Meis and Carvalho, 1974; Moreno-Sánchez, 1985), distribution of $[^3H]TPP^+$ (Moreno-Sánchez *et al.*, 1995), and matrix pH (Rodríguez-Zavala and Moreno-Sánchez, 1998) were measured by previously described procedures. Mitochondrial and cellular protein was determined by the biuret method in the presence of 1% (w/v) sodium deoxycholate, using bovine serum albumin as standard. To avoid interference in the protein determination by turbidity from glycogen, the tubes containing the reaction mixture were centrifuged at 3000 rpm for 5 min prior to measuring absorbance; however, glycogen turbidity did not alter the protein determination.

Determination of Total Fatty Acids

This was made following the procedure described by Folch and Sloane-Stanley (1957). Aliquots of 10 mg mitochondrial protein were stored at −70°C with 500 μg butyl hydroxytoluene (BHT) until use. After thawing, 1 ml BHT in 0.02% (v/v) methanol, 60 μg margaric acid (as standard), 1 ml 0.9% NaCl, and 2 ml chloroform were added and mixed for 1 min. The suspension was centrifuged at $1600 \times g$ for 10 min at 4°C. The organic phase was recovered and the aqueous phase was extracted two or three times more with chloroform. The organic phase was dehydrated by addition of 1.5 g Na_2SO_4 anhydrous. The solvent was evaporated under a stream of N_2. For *trans*-esterification of phospholipids and derivatization of free fatty acids to methyl esters (Christie, 1989), dried samples were mixed with 0.1 ml toluene, 2 ml methanol anhydrous, and 0.04 ml sulfuric acid. This mixture was heated at 65°C for 2 h. Then, the samples were mixed with 1 ml 5% NaCl and 2 ml hexane, and stirred vigorously, evaporated under N_2 stream, and stored at −72°C until gas chromatography (GC) analysis was carried out. The conditions for GC were

198°C, with H_2 as carrier at a pressure of 0.5 kg cm^{-2}. The column used was CP SIL 8CB in a Carlo Erba (Chromopack) instrument.

Content of Cytochromes

Absorbance difference spectra of mitochondria (2 mg protein/ml) were carried out in a medium that contained 60 mM KCl, 10 mM MOPS, 0.25 mM EGTA, pH 7.2, and 50% (v/v) glycerol. The baseline (oxidized *minus* oxidized) was recorded with the mitochondrial samples kept under 100% O_2 gassing during the acquisition of the spectrum. The reduction of the mitochondrial suspension in the experimental cuvette by dithionite, allowed the measurement of the reduced *minus* oxidized spectrum. The absorbance difference between 605 *minus* 630 nm was used for the calculations of the content of cytochrome $a + a_3$, using an extinction coefficient of 26.4 mM^{-1} cm^{-1} (Inamoto *et al.*, 1994). The mixture in the experimental cuvette was also reduced with 10 mM ascorbate (+2 mM azide). The spectrum of ascorbate-reduced versus oxidized was used to calculate the content of cytochrome $c_1 + c$, using the differences in absorbance at 550 *minus* 540 nm and an extinction coefficient of 19 mM^{-1} cm^{-1} (Inamoto *et al.*, 1994). To estimate the content of cytochrome *b*, without the interference of cytochromes $c_1 + c$, the absorbance difference at 563 *minus* 578 nm in the spectrum of dithionite-reduced versus ascorbate-reduced was measured; the extinction coefficient used was 17.9 mM^{-1} cm^{-1} (Inamoto *et al.*, 1994).

Determination of the Matrix Ion Content

Thawed aliquots of mitochondria (10 mg protein), previously washed with 0.25 M sucrose, 10 mM HEPES, and 1 mM EGTA, pH 7.2, were digested by boiling in 0.5 ml of 99% (v/v) nitric acid for 2 h in a block heater. Metals were measured in the transparent digested solution in an atomic absorption spectrophotometer (Pye-Unicam SP 192).

Content of Adenine Nucleotides

A small piece of liver was quickly removed from pentobarbital-anesthesized rats and immediately frozen in liquid N_2. After grinding in a cold mortar in a medium that contained 1 mM EGTA, 1 mM DTT, and 0.2% (w/v) albumin, pH 7.4, the tissue was mixed with 3% (w/v) perchloric acid *plus* 20 mM EDTA (Wanders *et al.*, 1984). Aliquots of freshly prepared mitochondria (10 mg protein) were also treated with perchloric acid +EDTA. These mixtures were centrifuged to remove denatured protein. The supernatant was treated with a 1:1 mixture of 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine, to remove perchlorate (Khym, 1975). The neutralized samples were used to determine ATP, ADP, and AMP by standard enzymic assays (Bergmeyer, 1983).

Western Blot Analysis

Immunodetection (Towbin *et al.*, 1979) of UCP2 in liver mitochondria was made by using a commercial antibody developed against an amino terminus epitope of human UCP2, which is conserved in the rat (Santa Cruz Biotech., Cat. Num sc-6526). Briefly, 60 μg of liver mitochondrial protein from control and cold-treated rats were resolved by a 15% PAGE–SDS electrophoresis. Thereafter, the proteins were electroblotted into a PVDF membrane. The membrane was blocked by incubation with 5% nonfat milk in TBST (0.1 M Tris, 0.13 M NaCl, 0.1% Tween 20, pH 7.6) for 1 h at room temperature and further incubated overnight at 4°C with the UCP2 antibody (1:1000 dilution). After rinsing the membrane twice with TBST for 15 min, a more stringent wash with 1 M NaCl TBST for 10 min was made, to ensure a high antibody specificity. The membrane was then incubated 2 h with a donkey anti-goat secondary antibody coupled to horseradish peroxidase (1:4000 dilution). The membrane was washed again with TBST and UCP2 detection was performed by a chemiluminiscent reaction as described by the manufacturer (ECL, Amersham). This was followed by densitometric analysis of the detected proteins.

RESULTS

Respiration and ATP Synthesis

Rats were exposed to cold (4°C) for up to 1000 h. At various times, liver mitochondria were prepared and their State 4 (basal) and State 3 (ADP-stimulated) rates of respiration were determined with 2-oxoglutarate (2-OG) (Fig. 1A) and succinate in the presence of rotenone (Fig. 1B) as substrates. With both substrates, mitochondria from cold-exposed rats exhibited higher respiratory rates than those from control animals. With 2-OG, three phases were distinguished: an initial phase in the first 150 h, in which respiration increased proportionally; thereafter, respiratory rates remained constant for about 200 h. This phase was followed by a second increase in respiratory rates (Fig. 1A). Similar patterns were observed with other

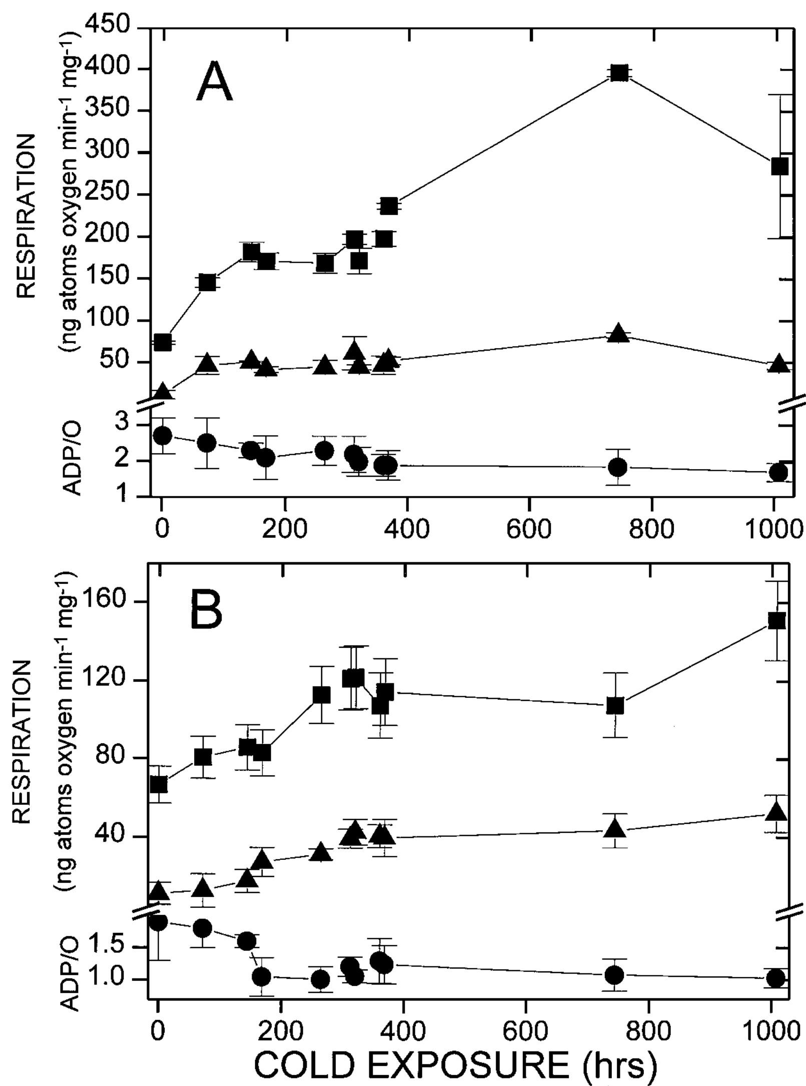

Fig. 1. Effect of cold stress on respiratory rates of rat liver mitochondria. Mitochondria, isolated from cold exposed rats for the indicated times, were incubated at a concentration of 1 mg protein/ml at 34°C. The incubation medium contained 120 mM KCl, 20 mM MOPS, 1 mM EGTA, 2 mM K-phosphate, 1 mM $MgCl_2$, pH 7.25, and 10 mM 2-oxoglutarate (A) or 5 mM succinate + 1 μM rotenone (B). The basal rate of respiration was measured (triangles) for approximately 2 min; then, 0.2–0.5 mM ADP was added to generate State 3 respiration (squares). Similar rates of basal respiration were obtained in the absence or in the presence of 1 μM oligomycin. The ADP/O ratios (circles) were calculated from the amount of ADP added in nanomoles and the extra amount of nanogram atoms of oxygen consumed by addition of ADP. The data shown represent the mean ± SD of at least 15 different mitochondrial preparations.

NAD-linked substrates, such as 10 mM glutamate, 5 mM pyruvate, 10 mM isocitrate, 10 mM citrate, 25 μM DL-palmitoyl-carnitine, and 50 μM octanoate (+2 mM malate) (data not shown). The ADP/O ratios significantly diminished ($p < 0.005$) from 2.8 to 1.6 with 2-OG and from 1.9 to 1.1 with succinate after 1000 h of cold stress (Fig. 1). The respiratory control values diminished from 5 to 1.9 with 2-OG and from 5.6 to 2.8 with succinate after 380 h of cold exposure. These results indicated that cold stress affected respiration through a mechanism that is not associated with stimulation of a specific enzyme or a metabolic pathway. Instead, the findings suggest that cold induces classical uncoupling of oxidative phosphorylation.

When the latter possibility was explored, it was found that the rate of ATP synthesis decreased by exposure to cold. Cold exposure for 280 or 380 h induced a slight, but significant diminution in ATP synthesis ($p < 0.001$) with 2-OG as substrate (Fig. 2); with succinate the decrease in ATP synthesis was more severe after 48 h of cold exposure. These changes in the rate of ATP synthesis were accompanied by increases of six and two times in the rate of respiration with 2-OG and succinate, respectively (Fig. 2). Similar relationships to those obtained with succinate were observed for glutamate, pyruvate, citrate, isocitrate, palmitoyl-carnitine, and octanoate (+ malate) (data not shown). It is noted, however, that in the first 24 h of cold exposure, there was a significant stimulation of oxidative phosphorylation with 2-OG (Fig. 2).

Surprisingly, the flow-force relationships revealed that exposure to different times of cold exerted a negligible effect on membrane potential, albeit there were significant variations in ATP synthesis (Fig. 3A and B) and respiration (Fig. 3C and D). The pH gradient, calculated from the direct measurement of the matrix pH in BCECF-loaded mitochondria, was lower than 0.1 in control mitochondria and 0.14 ± 0.04 ($n = 3$) in cold-stressed mitochondria under all conditions of Fig. 3. Taken together, these findings indicated that, mechanistically, classical uncoupling could not account for the effects of cold stress on liver mitochondria.

Flux Control Coefficients

The effect of cold exposure on the control of respiration, the flux control coefficients of the respiratory chain and the H^+ leak was determined using the elasticity-based analysis (Kacser and Burns, 1973; Groen *et al.*, 1986; Moreno-Sánchez *et al.*, 1999). Parallel titrations of

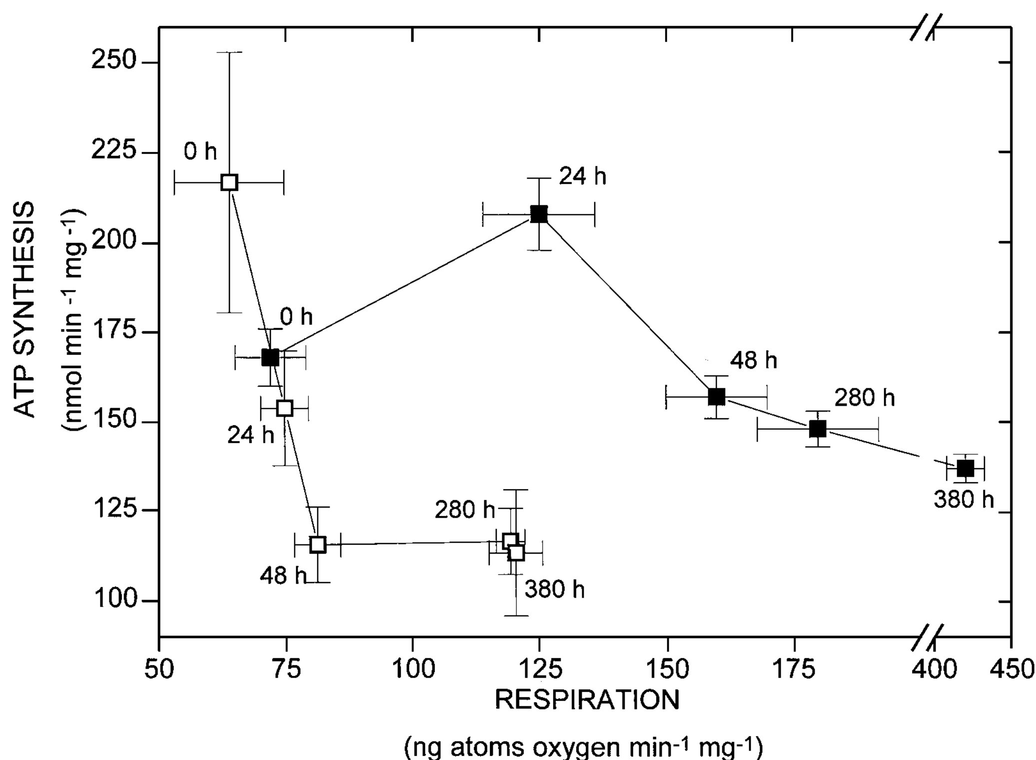

Fig. 2. Effect of cold stress on the respiration–ATP synthesis relationship. The rate of State 3 respiration was measured as described in the legend to Fig. 1. In parallel experiments, the rate of ATP synthesis was determined by the incorporation of ^{32}Pi into ATP. Mitochondria were incubated in the medium described in Fig. 1, which also contained ^{32}Pi (1×10^6 cpm/μmol), 10 mM glucose, 5 units hexokinase, and 10 mM 2-oxoglutarate (filled squares) or 5 mM succinate +1 μM rotenone (unfilled squares). After 2 min, 1 mM ADP was added to initiate ATP synthesis, which was allowed to proceed for 2 min; the reaction was stopped by addition of ice-cold 5% (w/v) trichloroacetic acid. Denatured protein was removed by centrifugation; the supernatant was mixed with ammonium molybdate + sulfuric acid to extract the remaining P_i with n-butylacetate + acetone (De Meis and Carvalho, 1974). The Cerenkov radiation of the washed aqueous phase was measured. The data are mean ± SD of six different mitochondrial preparations.

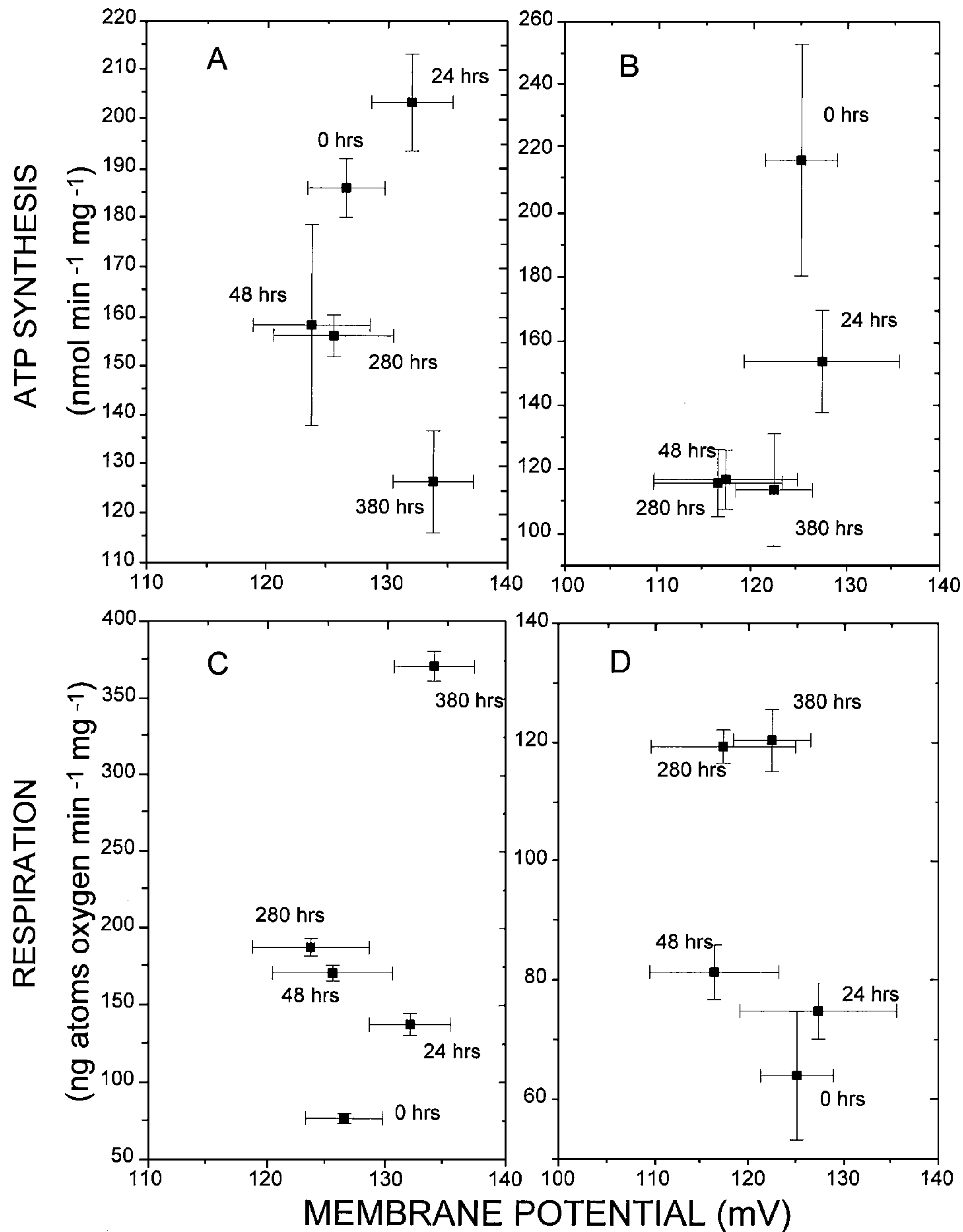

Fig. 3. Mitochondrial force-flux relationships under cold stress. The distribution of [^{3}H]TPP$^+$ across the inner mitochondrial membrane was used to determine the difference of transmembrane electric potential of mitochondria isolated from rats exposed to the indicated periods of cold. Mitochondria (2–3 mg protein/ml) were incubated at 34°C in the same medium described in Fig. 1 with 0.8 μM [^{3}H]TPP$^+$ (60,000–70,000 cpm/nmol) and 10 mM 2-oxoglutarate (A and C) or 5 mM succinate + 1 μ M rotenone (B and D). After 5 min, 2 mM ADP was added. The reaction was stopped by fast centrifugation in a refrigerated microcentrifuge. The radioactivity of the pellet and the supernatant was measured to calculate the membrane potential of control and cold-stressed mitochondria, using the Nernst equation and the corrections for nonspecific binding described by Rottenberg for rat liver mitochondria (Rottenberg, 1984). In parallel experiments with the same preparations, the rates of respiration and ATP synthesis were measured as described in the legends to Figs. 1 and 2, respectively. The data shown represent the mean ± SD of three different mitochondrial preparations.

the rate of respiration and the membrane potential with malonate or the uncoupler CCCP, in mitochondria from control and 380 h cold-stressed animals, were made at 34°C using succinate (+ rotenone) as substrate. The slopes of the titration curves were used to calculate the elasticity coefficients of the respiratory chain ($\varepsilon^{RC}_{\Delta\psi}$) and the H^+ leak ($\varepsilon^{L}_{\Delta\psi}$) toward the membrane potential. This, in turn, permits the estimation of the flux control coefficients by using the connectivity (1) and summation (2) theorems of the metabolic control analysis (Kacser and Burns, 1973; Groen *et al.*, 1986; Moreno-Sánchez *et al.*, 1999).

$$\varepsilon^{L}_{\Delta\psi} C_L + \varepsilon^{RC}_{\Delta\psi} C_{RC} = 0 \quad (1)$$

$$C_L + C_{RC} = 1 \quad (2)$$

The flux control coefficients of the H^+ leak (C_L) and the respiratory chain (C_{RC}) were $C_L = 0.67 \pm 0.1$ ($n = 5$) and $C_{RC} = 0.33 \pm 0.1$ (5) for control mitochondria and $C_L = 0.80 \pm 0.09$ ($p < 0.05$ versus control; $n = 5$) and $C_{RC} = 0.20 \pm 0.09$ ($n = 5$) for 380 h cold-stressed mitochondria.

Cold-induced Cytochrome and Metabolite Changes

After 48 h of cold stress, the content of cytochromes $c + c_1$ and b decreased and that of cytochromes $a + a_3$ increased (Table I). Thereafter, cytochromes $a + a_3$ returned to basal levels, whereas the contents of cytochromes $c + c_1$ and b followed an oscillatory pattern. The content of cytochrome b, however, reached a constant high level after 216–312 h of exposure to cold. This implies that the sole increase of respiratory rates cannot be due to an increase in cytochrome content. The activities of cytochrome c oxidase in liver homogenates at 30°C (in ng atoms oxygen mg protein^{-1} min^{-1}) were 294 ± 22 ($n = 5$), 455 ± 29 ($n = 5$), and 280 ± 10 ($n = 3$) for control, 133 and 380 h cold-stressed rats, respectively. Since the content of cytochromes $a + a_3$ was not altered in isolated mitochondria (Table I), the enhanced cytochrome c oxidase activity in the whole liver after 133 h of cold exposure suggests an increase in the cellular number of mitochondria, which returns to basal levels after 380 h.

Table I. Cytochromes Content in Liver Mitochondria (pmol/mg Protein) from Rats Exposed to Cold[a]

Cold exposure (h)	$c + c_1$	b	$a + a_3$
0 (5)	203 ± 36	211 ± 8.5	87.5 ± 14
48 (5)	148 ± 27[b]	176.5 ± 17[c]	124 ± 10.5[c]
75 (10)	161 ± 10[c]	243 ± 11[c]	82 ± 20
96 (5)	167 ± 8.5[d]	132 ± 22.5[c]	72 ± 40
144 (5)	262 ± 30[b]	139.5 ± 28[c]	79 ± 17
216 (5)	121 ± 40[e]	286 ± 26[c]	90 ± 16
278 (10)	203 ± 18	262 ± 40[b]	100 ± 21
312 (5)	211 ± 9.5	286 ± 39[c]	90 ± 11.5
360 (5)	170 ± 15.5	177 ± 25[b]	91.5 ± 12
576 (5)	208 ± 10	220 ± 17	79.5 ± 15
672 (5)	139.5 ± 26[b]	119 ± 32[c]	57.5 ± 22.5[d]

[a]Number of mitochondrial preparations assayed in parentheses. Values are mean ± SD. Student's t-test for nonpaired samples.
[b]$p < 0.025$ versus zero hours.
[c]$p < 0.005$.
[d]$p < 0.05$.
[e]$p < 0.01$.

The intramitochondrial contents of ATP and ADP varied reciprocally during exposure to cold (Fig. 4). The ATP/ADP ratio decreased, from an initial value of 1.0 to 0.1 after 350 h cold, indicating a diminished energy state due to decoupling. The AMP content increased with the time of cold exposure, but the total content of matrix adenine nucleotides remained constant. Values of the mitochondrial content of adenine nucleotides and cytochromes, for control rat liver mitochondria, similar to those reported here have been previously described (Gellerfors *et al.*, 1981; Wanders *et al.*, 1983; Inamoto *et al.*, 1994). The low mitochondrial energy state induced by cold stress was accompanied by a lowering in the liver content of ATP from 2.4 ± 1 to 0.16 ± 0.16 nmol/mg protein ($n = 3$) in 360 h cold-stressed livers. Although fast tissue removal and liquid N_2 freezing might be an ineffective procedure to prevent rapid changes in cellular ATP, the ATP levels found here are comparable to values obtained in liver tissue freeze-clamped with tongs precooled in liquid N_2 (Inamoto *et al.*, 1994). Thereby, these ATP levels reflect the difference in the energy state of the liver in the two conditions analyzed.

Exposure to cold diminished the content of total (esterified plus free) fatty acids in the mitochondrial membranes (Table II). In particular, αC18:3n-3 (γ-linolenic acid) decreased severely (3 times) after 380 h cold ($p < 0.005$). However, the relationships of palmitoleic/palmitic, oleic/stearic, γ-linolenic/linolenic, and saturated/insaturated fatty acids were not significantly modified by cold stress. The only significant decrease ($p < 0.05$) was for the araquidonic acid/dihomo-γ-linolenic acid relationship after 380 h cold, from 22.8 ± 4.6 (5) to 14.5 ± 7.5 (5) (mean ± SD; n).

Searching for other secondary changes induced by cold stress, such as osmoregulatory perturbations, the content of several intramitochondrial ions was determined. Ca^{2+} and Na^+ were not modified, Mg^{2+} and K^+ increased, and Zn^{2+}, Fe^{3+}, and inorganic phosphate decreased by cold stress (Table III). Note that the content of Fe^{3+} was much higher than that of cytochromes. This suggests that

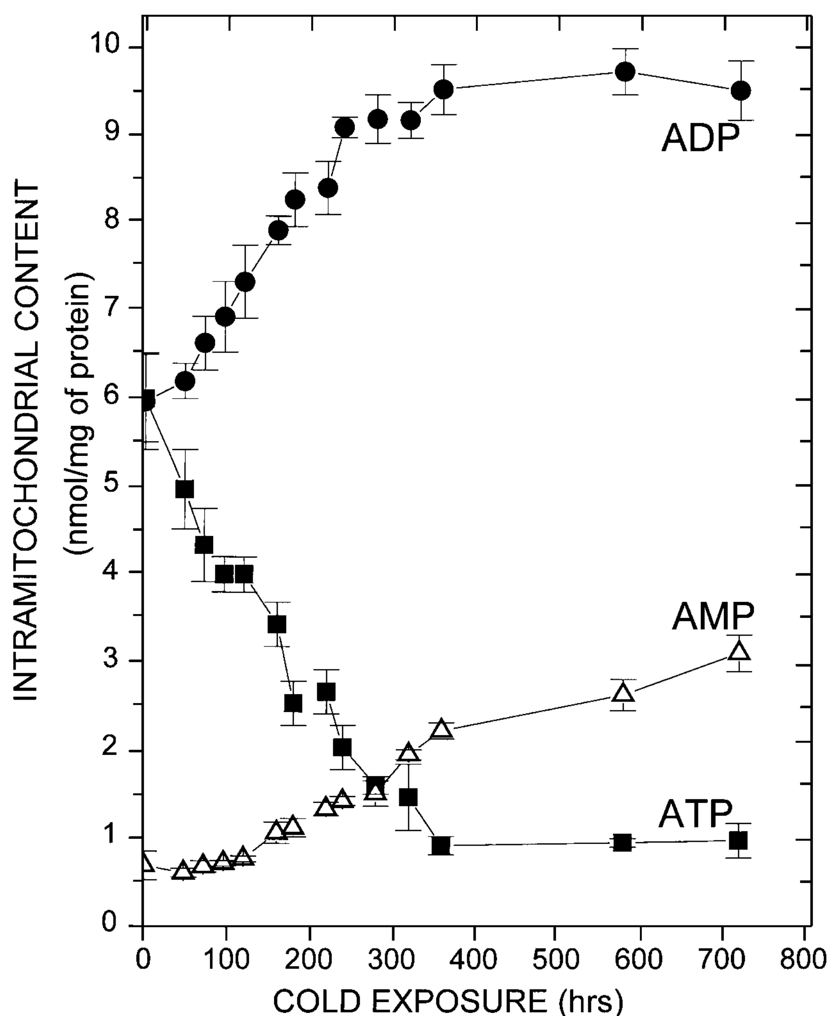

Fig. 4. Intramitochondrial content of adenine nucleotides at different times of cold exposure. The content of adenine nucleotides was determined as described under Methods. The data shown represent the mean ± SD of five different mitochondrial preparations.

excess Fe^{3+} in the mitochondrial matrix could be involved in the generation of reactive oxygen species. The iron content found for control mitochondria was also in the range of values reported in yeast mitochondria (Babcock *et al.*, 1997; Foury and Cazzalini, 1997). The diminution in matrix zinc and iron, during cold exposure, could be part of a protective mechanism against enhanced levels of oxygen reactive species in mitochondria with stimulated rates of basal respiration.

UCP2 and FFA Involvement in Mitochondrial Decoupling Induced by Cold

The presence of FFA and uncoupling proteins may be involved in the increased rate of respiration induced by cold stress. Since UCP2 is the only uncoupling protein present in liver mitochondria (Matsuda *et al.*, 1997; Kimura *et al.*, 1999; Ricquier and Bouillaud, 2000), and GDP may act as an inhibitor of UCP2 activity (Negre-Salvayre *et al.*, 1997; Jaburek *et al.*, 1999), the effect of GDP on the rate of respiration was assayed. GDP inhibited the respiration of cold-stressed mitochondria with 2-OG, but respiration of control mitochondria was not affected (Table IV). Addition of fatty acid-free albumin also diminished the respiratory rate in cold-stressed mitochondria, presumably due to the binding of FFA from the inner mitochondrial membrane.

Since classical uncoupling cannot satisfactorily explain the FFA effect on the H^+ gradient and fluxes (cf. Fig. 3), it is possible that FFA increased respiration

Table II. Content of Total Fatty Acids (μg/mg Protein) in Liver Mitochondria from Rats Exposed to Cold[a]

Fatty acid	Cold exposure (h)					
	0	24	48	120	280	380
C16 : 0	124.5 ± 18	97 ± 7[b]	88 ± 8[c]	67 ± 11[c]	94 ± 4[d]	86 ± 20[d]
C16 : 1	2.6 ± 0.6	2.6 ± 0.3	1.2 ± 0.5[c]	1.3 ± 0.3[c]	2.3 ± 0.8	2.3 ± 0.2
C18 : 0	225 ± 31.5	168.5 ± 6[c]	129 ± 7.5[c]	134 ± 6[c]	184.5 ± 16[e]	168 ± 12[c]
C18 : 1	70 ± 4	55 ± 3[c]	58 ± 6[c]	30.6 ± 4[c]	26 ± 12[c]	50 ± 18[e]
C18 : 2*n*-6	101 ± 9	81 ± 10.5[d]	68 ± 7[c]	68 ± 14[c]	91 ± 14.5	74 ± 11[c]
γ C18 : 3*n*-6	3.1 ± 0.4	1.7 ± 0.7[c]	1.4 ± 0.4[c]	1.3 ± 0.7[c]	1.4 ± 0.7[c]	1.8 ± 0.5[c]
α C18 : 3*n*-3	16 ± 6	11 ± 8	8.6 ± 2.3[b]	3.4 ± 1.8[c]	5.1 ± 1.9[c]	5.2 ± 2.3[c]
C20 : 1*n*-9	3.2 ± 1.8	2.4 ± 1.3	1.0 ± 0.6[b]	1.1 ± 0.4[e]	1.9 ± 0.4	2 ± 0.8
C20 : 3*n*-6	7 ± 4	8 ± 12	2.9 ± 2.7	4.3 ± 1.6	5.8 ± 1.6	7.5 ± 1.2
C20 : 4*n*-6	160 ± 18.5	123.5 ± 6[c]	97.5 ± 2.5[c]	95 ± 6[c]	100 ± 7[c]	109 ± 13[c]
C20 : 5*n*-3	7.2 ± 4.3	8 ± 6	2.9 ± 4	2.9 ± 6	7.2 ± 2.7	10.4 ± 2
C22 : 6*n*-3	84 ± 9.5	63 ± 10[d]	39.5 ± 9[c]	41.5 ± 6[c]	56 ± 7[c]	73 ± 3[e]
Total	815 ± 108	639.5 ± 60[b]	415 ± 50[c]	454 ± 57[c]	490 ± 69[c]	540 ± 85[c]

[a] Mean ± SD of five mitochondrial preparations.
[b] $p < 0.025$ versus 0 h.
[c] $p < 0.005$.
[d] $p < 0.01$.
[e] $p < 0.05$.

and decreased ATP synthesis by directly inducing decoupling, and/or by stimulating the activity of uncoupling proteins. To distinguish between these two possibilities, the effect of oleic acid and GDP was assayed. The addition of oleic acid increased the respiratory rate of control and cold-stressed mitochondria by 46–48 ng atoms oxygen/mg protein/min (Table IV). GDP decreased the rate of oleic acid-stimulated respiration by 34 ng atoms oxygen/mg protein/min in cold-stressed mitochondria and by 16.5 ng atoms oxygen/mg protein/min in control mitochondria. This suggests that there is a higher contribution of uncoupling proteins to the oleic acid-stimulated respiration in cold-stressed mitochondria than in control mitochondria.

The partial inhibitory effect of CAT on the basal rate of respiration in cold-stressed mitochondria, and on oleic acid-stimulated respiration in both types of mitochondria (Table IV), suggested the participation of the adenine nucleotide translocator in the effects of FFA (Brustovestsky *et al.*, 1990, 1992, 1993; Schonfeld, 1990). The content of the translocator, estimated from titrations of the rate of State 3 respiration with CAT, was essentially the same in

Table III. Mitochondrial Ion Content (nmol/mg Protein) at Different Times of Cold Exposure[a]

Cold exposure	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	Zn^{2+}	Fe^{3+}	P_i
0 (8)	16.5 ± 4	24 ± 9	10.5 ± 3	126.4 ± 9	18 ± 5	1.5 ± 0.1	28 ± 3
48 (5)	15 ± 4	29 ± 12	10.5 ± 2	115 ± 6[c]	14.6 ± 3	0.96 ± 0.02[d]	27.4 ± 3
75 (10)	15 ± 5	33 ± 4[b]	11.4 ± 3	197 ± 10[d]	11.5 ± 3[d]	0.88 ± 0.03[d]	30 ± 4
96 (5)	18 ± 2	38 ± 8[c]	11 ± 6	238 ± 7[d]	12 ± 2[c]	0.8 ± 0.5[d]	19 ± 4[d]
144 (5)	15 ± 6	39.5 ± 7[b]	10 ± 5	333 ± 8[d]	14.6 ± 4	0.8 ± 0.3[d]	22 ± 4[b]
216 (5)	15 ± 5	38 ± 8[c]	10 ± 6	321 ± 9[d]	11 ± 3[c]	0.9 ± 0.7[c]	29 ± 2
278 (10)	14.5 ± 4	40 ± 5[d]	11 ± 5	323 ± 9[d]	7 ± 4[d]	0.8 ± 0.6[d]	21 ± 3[d]
312 (5)	14 ± 6	35 ± 11[e]	11 ± 4	350 ± 8[d]	13 ± 4	0.8 ± 0.4[d]	22 ± 4[c]
360 (5)	14 ± 8	40 ± 6[d]	11 ± 2	336 ± 17[d]	10 ± 4[b]	0.8 ± 0.6[b]	22 ± 3[d]
576 (5)	16 ± 6	39.5 ± 9[b]	11 ± 3	318 ± 6[d]	9 ± 3[d]	0.9 ± 0.6[c]	
672 (3)	16 ± 7	47 ± 6[d]	11 ± 3	349 ± 7[d]	9 ± 4[c]	0.8 ± 0.9[e]	24 ± 5

[a] Mean ± SD with the number of mitochondrial preparations assayed in parenthesis.
[b] $p < 0.01$ versus 0 h.
[c] $p < 0.025$.
[d] $p < 0.005$.
[e] $p < 0.01$.

Table IV. Effect of Albumin, GDP, and CAT on Basal and Oleic-Stimulated Respiration (ng Atoms Oxygen min^{-1}/mg^{-1})[a]

	Control				380 h Cold			
	Basal (State 4)	+0.2% BSA	+3 mM GDP	+1 μM CAT	Basal (State 4)	+0.2% BSA	+3 mM GDP	+1 μM CAT
Pyr + Mal	13.5 ± 1	17 ± 3	17.5 ± 2.5	18 ± 1[c]	43 ± 6	30 ± 2[d]	36 ± 4	37 ± 3
2-OG	17.5 ± 2	18 ± 2	23 ± 6	22 ± 4	51 ± 7	29 ± 2[c]	36 ± 4[d]	40 ± 1[b]
Succ (+ rote)	17 ± 2	18 ± 2	19 ± 2	17.5 ± 2	36 ± 5	30 ± 5	30 ± 5	29 ± 3[e]

Succ (+ rote)	Basal (State 4)	50 nmol/mg protein Oleic acid	Oleic acid + BSA	Oleic acid + CAT	Oleic acid + GDP
Control	20 ± 4	66.5 ± 11	15 ± 9[f]	37 ± 11[f]	50 ± 8[f]
380 h Cold	35 ± 5	83 ± 14	23 ± 8[f]	44 ± 13[f]	49 ± 10[f]

[a]Mean ± SD of four mitochondrial preparation. 2-OG, 2-oxoglutarate; succ, succinate; rote, rotenone; BSA, bovine serum albumin; CAT, carboxy-atractyloside.
[b] $p < 0.025$ versus basal respiration.
[c] $p < 0.005$.
[d] $p < 0.01$.
[e] $p < 0.05$.
[f] $p < 0.005$ versus oleic acid-stimulated respiration.

mitochondria from control and 380 h cold-exposed animals (298 ± 25; $n = 6$; versus 312 ± 25; $n = 5$; pmol/mg protein, respectively).

Immunodetection analysis, using a commercial UCP2 antibody, showed low basal levels of a protein of approximately 35 kDa, the putative UCP2, in control liver mitochondria (Fig. 5A) and BAT mitochondria (not shown). Increments in this protein of 2.1 and 3.6 times were observed after 120 and 360 h of cold exposure; a longer cold exposure did not further enhance the protein content (Fig. 5B). Significant differences were found for these times versus zero h ($p < 0.05$). A second protein of 28 kDa was also occasionally detected by the UCP2 antibody (not shown).

DISCUSSION

Metabolic Changes Induced by Cold

The lack of change in the level of fatty acids insaturation in cold stress suggested that there was no modification of the fluidity of the mitochondrial inner membrane. Likewise, the activity of several liver fatty acid desaturases were not modified by cold stress, that is, the relationships of the relevant fatty acid product/substrate were not altered, except for a significant decrease in the araquidonic acid/dihomo-γ-linolenic acid ratio. Because the enzyme Δ-5 desaturase transforms dihomo-γ-linolenic acid into arachidonic acid, the observation suggests that the activity of this enzyme might be diminished during prolonged cold exposure.

Mak *et al.* (1983) essentially described the same fatty acid profile for liver mitochondria from control and 3- to 4-week cold-stressed rats. However, they reported percentage variations instead of absolute units; thus, it might be that under their conditions there was a diminution in total fatty acids similar to the one we observed (Table I). In this regard, the observation that albumin decreased respiration in mitochondria from cold exposed animals to values comparable to those of control mitochondria is mechanistically relevant. This very likely indicates that FFA levels are increased in cold-stressed mitochondria, although there is a general diminution in total fatty acid content.

Aithal and Ramasarma (1971) and Mak *et al.* (1983) previously reported that cold exposure did not affect the ADP/O ratio in liver mitochondria. Aithal and Ramasarma (1971) determined the actual P/O ratio by measuring extramitochondrial ATP with a hexokinase trap; however, with this method ATP levels may be overestimated. In addition, these authors reported unusual low values of ATP synthesis, which were increased by cold exposure. With respect to the work of Mak *et al.* (1983), they exposed the animals to 8°C not to 4°C; this suggests that the temperature used to induce cold stress may be relevant in the induction of mitochondrial metabolism changes. Goglia *et al.* (1988) described an increased rate of respiration in the light mitochondrial fraction, isolated from livers of rats exposed for 15 days at 4°C; however, their

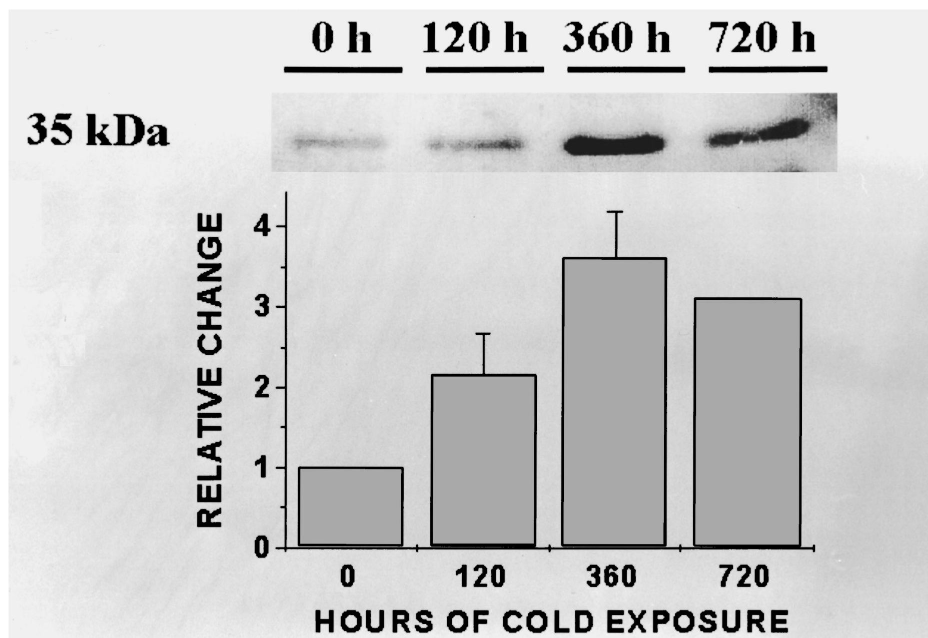

Fig. 5. UCP2 immunodetection in mitochondrial preparations of cold-exposed rats. (A) Immunodetection of UCP2 in mitochondria isolated from rats exposed to the indicated times of cold. (B) Densitometric analysis of immunoblots. The values shown represent the mean of three independent mitochondrial preparations assayed by duplicate, except for 720 h where only two preparations were analyzed. Levels of UCP2 are expressed as the ratio of the Western blot signal density/Ponceau stained protein amount (± standard error of the mean) for each lane.

respiration values were also unusually low. In contrast, our data (cf. Figs. 1 and 2) indicated that cold stress induced a significant diminution in the ADP/O ratios and in the rate of ATP synthesis. Our observed rates of respiration and oxidative phosphorylation were high and within the range of values reported for liver mitochondria by several groups.

Cold exposure for 24 h induced a significant increase ($p < 0.001$) in the rate of ATP synthesis (cf. Fig. 2A) and in the magnitude of the steady-state membrane potential (Fig. 3A), supported by 2-OG oxidation. Such activation was not obtained with glutamate + malate or any other oxidizable substrate. This specific activation could be related to an increase in the activities of the 2-oxoglutarate dehydrogenase complex and the 2-oxoglutarate carrier, induced by a short exposure to cold. This could be due to a net rise in the enzyme content or in the factors that modulate the enzyme complex activity. In this respect, further studies are required.

The increased respiration rate induced by cold exposure was accompanied by a diminution in the elasticity coefficient of the H^+ leak for the H^+ gradient and, hence, a closer saturation of this activity. In consequence, in liver mitochondria from cold-exposed rats, the H^+ leak exerts a degree of control on respiration higher than in control mitochondria. Similar flux control coefficients of the H^+ leak and the respiratory chain for succinate dependent respiration of control mitochondria from liver (Brand *et al.*, 1993) and brown adipose tissue (Monemdjou *et al.*, 1999) have been reported.

Role of UCP2 and FFA

In obesity due to leptin deficiency, there is an increase in respiration rate and diminished ATP synthesis, with no clear effect on the steady-state membrane potential in mouse liver mitochondria (Chavin *et al.*, 1999). These changes are similar to those observed here in mitochondria from cold-stressed rats. Immunohistochemical analyses have shown undetectable levels of the UCP2 in normal hepatocytes and high levels of this protein in Kupffer cells (Boss *et al.*, 1997; Chavin *et al.*, 1999). However, the metabolic changes in liver mitochondria from obese mice were accompanied by increases in the UCP2 mRNA and protein levels in hepatocytes (Chavin *et al.*, 1999). Here we found low levels of UCP2 in control liver mitochondria, but a significant enhancement in the putative UCP2 content after 120 h of cold exposure.

An increased UCP2 mRNA expression has been reported in lipopolysaccharide-treated (Cortez-Pinto *et al.*, 1998) and transformed (Fleury *et al.*, 1997) rat hepatocytes. Cold also induces enhanced expression of UCP2 mRNA in BAT, heart, and soleus skeletal muscle, but other tissues, including liver, have not been analyzed (Carretero *et al.*, 1998). These data suggest that UCP2 expression can be induced in response to different stimuli in liver. Thus, it is conceivable that cold stress promotes an increase in the expression of the UCP2 gene in Kupffer cells, in hepatocytes or in both.

Because UCP1 is only expressed in BAT (Ricquier and Bouillaud, 2000), and UCP3 is not expressed in liver (Matsuda *et al.*, 1997; Kimura *et al.*, 1999), the protein detected in this work by the commercial antibody is very likely the UCP2. Northern analysis using the different UCP probes in cold-stressed liver could further support this point. It is well established that UCP1 protonophoric activity is regulated by fatty acids and nucleotides (Nicholls and Locke, 1984; Rial *et al.*, 1983; Ricquier and Bouillaud, 2000), but it is controversial if UCP2 activity is also modulated by these ligands (Negre-Salvayre *et al.*, 1997; Jaburek *et al.*, 1999; Rial *et al.*, 1999; Ricquier and Bouillaud, 2000). However, the described inhibitory effect of GDP and albumin (i.e., binding of FFA) on respiration of cold-stressed mitochondria (Table IV) suggests that UCP2 might also be modulated by these ligands. Indeed, a correlation between the UCP2 content (Fig. 5) and the rate of respiration (Fig. 1) was attained.

The strong stimulatory effect on respiration and inhibition on ATP synthesis and ADP/O ratios induced by cold stress, with no associated diminution in the H^+ gradient, indicates that classical uncoupling is not involved in this process. Agents that uncouple oxidative phosphorylation without significant reduction in the bulk H^+ gradient are called "decouplers" (Rottenberg, 1990). Decouplers are assumed to inhibit energy-dependent fluxes by releasing H^+ from a putative intramembrane H^+ transfer pathway. FFA, at appropriate concentrations, may induce "decoupling" of oxidative phosphorylation (Rottenberg, 1990). Indeed, the marked diminution in respiration induced by albumin in cold-stressed mitochondria indicates the presence of FFA in the inner membrane of these mitochondria. The inhibitory effect of CAT on respiration also supports an active flux of the anionic fatty acid through the inner membrane in a reaction catalyzed by the adenine nucleotide translocase.

Therefore, the metabolic perturbations induced by cold stress in rat liver mitochondria seem to be caused by the presence of FFA in the inner membrane and an enhanced level of UCP2 which, by inducing decoupling, affects respiration, ATP synthesis, ion and metabolite contents, and H^+ leakage. The effect of UCP2 on the H^+ gradient is not clear (Chavin *et al.*, 1999) and, therefore, its mechanism of action may be either through uncoupling or decoupling. A local generation of heat, mediated by FFA and UCP2, may represent a possible biochemical adaptation mechanism to cold in rat liver mitochondria. Other functions of FFA and UCP2 such as control of the NAD^+ redox state, or of the levels of ATP and reactive oxygen species may also be hypothesized (Ricquier and Bouillaud, 2000).

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REFERENCES

Aithal, H. N. and Ramasarma, T. (1971). *Biochem. J.* **123**, 677–682.

Babcock, M., de Silva, D., Oaks, R., Davis-Kaplan, S., Jiralerspong, S., Montermini, L., Pandolfo, M., and Kaplan, J. (1997). *Science* **276**, 1709–1712.

Bakarat, H. A., Kasparek, G. J., Tapscott, L. B., and Snider, R. D. (1982). *Biochem. J.* **208**, 419–424.

Bergmeyer, H. U. (1983). In *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.), Vol. 4. Verlag Chemie, Weinheim.

Bode, A. M., Byrd, S., and Klug, G. A. (1990). *Biochim. Biophys. Acta* **1047**, 161–167.

Boss, O., Samec, S., Dulloo, A., Seydoux, J., Muzzin, P., and Giacobino, J. P. (1997). *FEBS Lett.* **412**, 111–114.

Brand, M. D., Harper, M. E., and Taylor, H. C. (1993). *Biochem. J*. **291**, 739–748.

Brustovestsky, N. N., Amerkanov, Z. G., Yegorova, M. E., Mokhova, E. N., and Skulachev, V. P. (1990). *FEBS Lett.* **272**, 190–192.

Brustovestsky, N. N., Egorova, M. V., Gnutov, D. Y., Gogvadze, V. G., Mokhova, E. N., and Skulachev, V. P. (1992). *FEBS Lett.* **305**, 15–17.

Brustovestsky, N. N., Egorova, M. V., Gnutov, D. Y., Mokhova, E. N., and Skulachev, V. P. (1993). *FEBS Lett.* **315**, 233–236.

Carretero, M. C., Torres, L., Latasa, M. U., Garcia-Trevijano, E. R., Prieto, J., Mato, J. M., and Avila, M. A. (1998). *FEBS Lett.* **439**, 55–58.

Cortez-Pinto, H., Yang, S. Q., Lin, H. Z., Costa, S., Hwang, C. S., Lane, M. D., Bagby, G., and Diehl, A. M. (1998). *Biochem. Biophys. Res. Commun.* **251**, 313–319.

Chávez, E., Moreno-Sánchez, R., Torres-Márquez, M. E., Zazueta, C., Bravo, C., Rodríguez-Enríquez, S., García, C., Rodríguez, J. S., and Martínez, F. (1996). *J. Bioenerg. Biomembr.* **28**, 69–76.

Chavin, K. D., Yang, S. Q., Lin, H. Z., Chatham, J., Chacko, V. P., Hoek, J. B., Walajtys-Rhode, E., Rashid, A., Cheu, C. H., Huang, C. C., Wu, T. C., Lane, M. D., and Diehl, A. M. (1999). *J. Biol. Chem.* **274**, 5692–5700.

Christie, W. W. (1989). The preparation of derivatives of fatty acids. In *Gas Chromatography and Lipids* (W. W. Christie, ed.). The Oily Press, Scotland, pp. 64–84.

De Meis, L. and Carvalho, M. G. C. (1974). *Biochemistry* **13**, 5032–5038.
Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Levi-Meyrueis, C., Bouillaud, F., Seldin, M. F., Surwit, R. S., Ricquier, D., and Warden, C. H. (1997). *Nature Genet.* **15**, 269–272.
Folch, L. M. and Sloane-Stanley, C. H. (1957). *J. Biol. Chem.* **226**, 497–509.
Foury, F. and Cazzalini, O. (1997). *FEBS Lett.* **411**, 373–377.
Gellerfors, P., Johansson, T., and Nelson, B. D. (1981). *Eur. J. Biochem.* **115**, 275–278.
Goglia, F., Liverini, G., Lanni, A., Iossa, S., and Barletta, A. (1988). *Biochem. Biophys. Res. Commun.* **151**, 1241–1249.
Grav, H. J. and Blix, A. S. (1979). *Science* **204**, 87–89.
Groen, A. K., Van Roermund, C. W. T., Vervoorn, R. C., and Tager, J. M. (1986). *Biochem. J.* **237**, 379–389.
Inamoto, T., Tanaka, A., Mori, S., Jin, M. B., Sato, B., Yanabu, N., Tokuka, A., Kitai, T., Ozawa, K., and Yamaoka, Y. (1994). *Biochim. Biophys. Acta* **1188**, 311–317.
Jaburek, M., Varecha, M., Gimeno, R. E., Dembski, M., Jezek, P., Zhang, M., Burn, P., Tartaglia, L. A., and Garlid, K. D. (1999). *J. Biol. Chem.* **274**, 26003–26007.
Kacser, H. and Burns, J. A. (1973). *Symp. Soc. Exptl. Biol.* **27**, 65–104.
Khym, J. X. (1975). *Clin. Chem.* **21**, 1245–1252.
Kimura, K., Dung, B. D., Kanehira, K., Irie, Y., Cañas, X., and Saito, M. (1999). *FEBS Lett.* **457**, 75–79.
Klug, G. A., Krause, J., Ostlund, A. K., Knoll, G., and Brdiczka, D. (1984). *Biochim. Biophys. Acta* **764**, 272–282.
Mak, I. T., Shrago, E., and Elson, C. E. (1983). *Biochim. Biophys. Acta* **722**, 302–309.
Matsuda, J., Hosoda, K., Itoh, H., Son, C., Doi, K., Tanaka, T., Fukunaga, Y., Inoue, G., Nishimura, H., Yoshimasa, Y., Yamori, Y., and Nakao, K. (1997). *FEBS Lett.* **418**, 200–204.
Monemdjou, S., Kozak, L. P., and Harper, M. E. (1999). *Amer. J. Physiol.* **276**, E1073–E1082.
Moreno-Sánchez, R. (1985). *J. Biol. Chem.* **260**, 4028–4034.
Moreno-Sánchez, R., Rodríguez-Enríquez, S., Cuéllar, A., and Corona, N. (1995). *Arch. Biochem. Biophys.* **319**, 432–444.
Moreno-Sánchez, R., Bravo, C., and Westerhoff, H. V. (1999). *Eur. J. Biochem.* **264**, 427–433.
Negre-Salvayre, A., Hirtz, C., Carrera, G., Cazenave, R., Troly, M., Salvayre, R., Penicaud, L., and Casteilla, L. (1997). *FASEB J.* **11**, 809–881.
Nicholls, D. G. and Locke, R. M. (1984). *Physiol. Rev.* **64**, 1–64.
Rial, E., González-Barroso, M., Fleury, C., Iturrizaga, S., Sanchís, D., Jiménez-Jiménez, J., Ricquier, D., Goubern, M., and Bouillaud, F. (1999). *EMBO J.* **21**, 5827–5833.
Rial, E., Poustie, A., and Nicholls, D. G. (1983). *Eur. J. Biochem.* **137**, 197–203.
Ricquier, D. and Bouillaud, F. (2000). *Biochem. J.* **345**, 161–179.
Rodríguez-Zavala, J. S. and Moreno-Sánchez, R. (1998). *J. Biol. Chem.* **273**, 7850–7855.
Rottenberg, H. (1984). *J. Membr. Biol.* **81**, 127–138.
Rottenberg, H. (1990). *Biochim. Biophys. Acta* **1018**, 1–17.
Scholz, R., Schwabe, U., and Soboll, S. (1984). *Eur. J. Biochem.* **141**, 223–230.
Schonfeld, P. (1990). *FEBS Lett.* **264**, 246–248.
Seitz, H. J., Muller, M. J., Krone, W., and Tarnowski, W. (1977). *Arch. Biochem. Biophys.* **183**, 647–663.
Skulachev, V. P. (1991). *FEBS Lett.* **294**, 158–162.
Soboll, S. and Stucki, J. (1985). *Biochim. Biophys. Acta* **807**, 245–254.
Soboll, S., Grundel, S., Schwabe U., and Scholz, R. (1984). *Eur. J. Biochem.* **141**, 231–236.
Towbin, H., Staehelin, T., and Gordon, J. (1979). *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
Wanders, R. J. A., Meijer, A. J., Groen, A. K., and Tager, J. M. (1983). *Eur. J. Biochem.* **133**, 585–589.
Wanders, R. J. A., Van den Berg, G. B., and Tager, J. M. (1984). *Biochim. Biophys. Acta* **767**, 113–119.