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Respiratory Failure and Stimulation of Glycolysis in Chinese Hamster Ovary Cells Exposed to Normobaric Hyperoxia*

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Continuous exposure of Chinese hamster ovary (CHO) cells to an atmosphere of 98% O_2, 2% CO_2 (normobaric hyperoxia) leads within a period of several days to cytostasis and clonogenic cell death. Here we report respiratory failure as an important early symptom of oxygen intoxication in CHO cells, resulting in a more than 80% inhibition of oxygen consumption within 3 days of hyperoxic exposure. This inhibition appeared to be correlated with selective inactivation of three mitochondrial key enzymes, NADH dehydrogenase, succinate dehydrogenase, and α-ketoglutarate dehydrogenase. The latter enzyme controls the influx of glutamate into the Krebs cycle and is particularly critical for oxidative ATP generation in most cultured cells, which depends on exogenous glutamine rather than glucose as a carbon source. As expected, the inactivation of α-ketoglutarate dehydrogenase was correlated with a fall in cellular glutamine utilization, which became apparent from the first day of hyperoxic exposure. Thereafter, glucose utilization and lactate excretion started to increase, up to 3-fold, indicating a cellular response to respiratory failure aimed at increased ATP generation from glycolysis. However, in spite of this response, the cellular ATP level progressively decreased, up to 2.5-fold. Thus, killing of CHO cells by normobaric hyperoxia seems to be due to a severe disturbance of mitochondrial metabolism eventually leading to a depletion of cellular ATP pools.

In multicellular organisms oxygen is indispensable for the production of energy and maintenance of the living state. Yet elevated oxygen levels (hyperoxia) are usually toxic (1–3). Oxygen toxicity is mediated by activated oxygen species, such as superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, which are produced as a result of normal aerobic metabolism (2–4). Under hyperoxic conditions, increased amounts of these radicals are formed, which overwhelm the cellular defense systems (4). In proliferating cell cultures, the toxicity of hyperoxia manifests itself in growth inhibition (5–9), reduced plating efficiency (10), and chromosomal breakage (11, 12).

The physiological target processes primarily involved in hyperoxic intoxication are unknown. Studies of hyperoxia-exposed tissue slices and homogenates have implicated membrane damage and inactivation of several key metabolic enzymes, especially those containing essential sulfhydryl-groups (1, 2, 4). However, those data do not necessarily reflect what happens in the intact cell, in which free radical generation and antioxidant defenses are optimally balanced due to compartmentalization. For cultured cells there is evidence that hyperoxia induces a disturbance of energy metabolism, *i.e.* lactate production is increased (5, 7, 8, 13), mitochondrial morphology is affected (9, 14), and ATP levels become depleted (15).

In the present study, an attempt was made to characterize the disturbance of energy metabolism in normobaric hyperoxia-exposed CHO[1] cells. This was done by polarographic measurements of oxygen consumption in intact as well as digitonin-permeabilized cells (16–18). The latter technique allows the separate measurement, on a *per cell* basis, of the activities of NADH (rotenone-sensitive) oxidase (complex I), succinate oxidase (complex II), and the α-glycerophosphate oxidase complex, without the necessity for mitochondrial purification. The same enzyme activities were also measured spectrophotometrically in homogenates. In addition, we also studied the α-ketoglutarate dehydrogenase complex, a NADH-generating regulatory enzyme that controls the influx of glutamine/glutamate into the Krebs cycle, which is the most important route for oxidative ATP generation in cell cultures (19–21). This enzyme complex consists of multiple copies of three enzymes, termed *E*1, *E*2, and *E*3. *E*1 is a 2-keto acid dehydrogenase, *E*2 is a dihydrolipoamide acyltransferase, and *E*3 is a dihydrolipoamide dehydrogenase, which also contains a NADH diaphorase activity (22). Early studies on tissue slices and homogenates have noted a high sensitivity to oxygen of both the entire complex and of *E*3 (Ref. 1), which was attributed to the presence of critical -SH groups in these enzymes.

The inhibition of respiratory activity during hyperoxic exposure led us to study also (i) the activity of key glycolytic enzymes, especially the hydrogen peroxide-sensitive sulfhydryl-containing enzyme glyceraldehyde-3-phosphate dehydrogenase (1, 2, 23), (ii) the activity of a NADPH recycling system, *i.e.* glucose-6-phosphate dehydrogenase and glutathione reductase (24), and (iii) the utilization of the energy sources glucose *versus* glutamine/glutamate (19–21).

The present data reveal that O_2 poisoning of CHO cells is characterized by an early and severe inactivation of the α-ketoglutarate dehydrogenase complex, along with a parallel

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[1] The abbreviations used are: CHO, Chinese hamster ovary; DNP, dinitrophenol.

but less severe inactivation of complexes I and II of the respiratory chain, eventually leading to a dramatic inhibition of mitochondrial ATP generation. The cells respond to this inhibition by shifting their metabolism toward an increased rate of glycolytic ATP synthesis. However, this shift apparently cannot prevent further depletion of ATP, so that continued hyperoxia inevitably leads to cell death.

MATERIALS AND METHODS

Chemicals—All chemicals were of analytical grade and obtained from Sigma or Boehringer (Mannheim, Federal Republic of Germany (F.R.G.)), except K_2HPO_4, KH_2PO_4, $MgCl_2$, $MgSO_4$, EDTA, and KCN, which were obtained from Merck (Darmstadt, F.R.G.), sucrose (Baker, Deventer, The Netherlands), potassium ferricyanide (Fluka, Buchs, Switzerland) and glucose and succinic acid (BDH Chemicals, Dorset, United Kingdom).

Cell Culture—CHO cells were cultured under an atmosphere of air, 2% CO_2 (normoxia) at 37 °C in 75-cm^2 sealed polystyrene flasks containing 30 ml of Ham's F-10 medium (Flow Lab. Ltd., Irvine, Scotland), supplemented with 1 mM L-glutamine and 10% heat-inactivated (30 min, 56 °C) fetal calf serum (Flow) (complete culture medium). The method of gassing the cultures was as described before (9). Cells were routinely subcultured twice a week by trypsinization and seeding at a starting density of 5×10^5 cells/flask. Culture medium pH was in the range of 6.8–7.4 at all times. Cultures were free from mycoplasma contamination, as checked at monthly intervals.

Experimental Protocol—To study the time course of oxygen poisoning in cultures of proliferating cells, in principle two procedures could be used: (i) to monitor the cultures in a sequential way by analyzing samples taken at daily intervals, or (ii) to shift cultures from normoxia to hyperoxia at daily intervals and harvest the cultures at a single point in time. The first method has several disadvantages, *i.e.* cultures are compared having largely different densities; day-to-day variation is added to biological variation; there are large differences in time interval from seeding to harvest, while freshly trypsinized cells seem to be hypersensitive to hyperoxia. Therefore, the second procedure was adopted, as outlined below.

CHO cells were seeded in multiple 75 cm^2 flasks (5×10^5 cells/flask) and allowed to proliferate under normoxic conditions over a period of 4 days. After 1, 2, and 3 days subseries of flasks were gassed with a hyperoxic atmosphere (98% O_2, 2% CO_2). All cells from one flask were harvested by trypsinization and suspended in 10 ml of complete culture medium, after which the cells were counted in a Coulter counter.

During the experiment plating efficiencies of all cultures were determined at daily intervals by seeding 400–800 cells into Petri dishes, which were incubated at 37 °C with 10 ml of culture medium under an atmosphere of air, 2% CO_2. After 1 week, colonies were fixed with acetic acid/methanol (1:3, v/v), stained with Giemsa, and counted manually.

In the experiments described below the effects of 1 and 2 days hyperoxic exposure time were examined at 3 days culturing time (*A* in Fig. 1); the effects of 1, 2, and 3 days hyperoxia were analyzed by studying the cultures at 4 days culturing time (*B* in Fig. 1).

Oxygen Consumption—Cells ($5–12 \times 10^6$) were trypsinized, suspended in 11 ml of complete culture medium, and counted. After centrifugation ($800 \times g$, 5 min) cells were resuspended either in buffer A (for measurements on intact cells), *i.e.* 100 μl of Tris/HCl (25 mM, pH 7.4), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), NaCl (110 mM), and KCl (6.7 mM), or in buffer B (for measurements on digitonin-permeabilized cells), *i.e.* 100 μl of Tris/HCl (25 mM, pH 7.4), 0.25 M sucrose, 2 mM EDTA, 5 mM $MgCl_2$, and 10 mM K_2HPO_4. Oxygen consumption of cells was recorded in a polarographic cell (0.89 ml) at 37 °C with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, OH). Permeabilization was achieved by adding digitonin (200 μM, 0.25 mg/10^7 cells), a concentration that permeabilizes the plasma membrane but leaves mitochondrial membranes intact (2, 17, 25). After addition of digitonin, recording was continued in the presence of malate (5 mM), rotenone (100 nM), succinate (5 mM), malonate (10 mM), α-glycerophosphate (5 mM), DNP (25 μM), and KCN (1 mM). These compounds were successively added from 100 to 200-fold concentrated stock solutions. NADH oxidase activity was calculated by subtracting from the respiration rate observed in the presence of malate the residual rate measured after addition of rotenone (an inhibitor of complex I), succinate oxidase activity was measured by taking the net respiration rate induced by the addition of succinate; α-glycerophosphate oxidase activity was measured as the respiration rate induced by α-glycerophosphate added after blocking the succinate oxidase activity with malonate.

Spectrophotometric Enzyme Assays—CHO cells were trypsinized and suspended in 10 ml of complete culture medium, followed by centrifugation ($800 \times g$, 5 min). The pellet was resuspended in 250 μl of ice-cold Tris/HCl (25 mM, pH 7.4), supplemented with 0.25 M sucrose and 2 mM EDTA, after which the suspension was sonicated with a sonifier (40,000 V, 3×20 s, with 40-s intervals). During the experiments, the lysates were kept on ice. All enzyme assays described below were carried out in a thermostated Shimadzu model UV-160 spectrophotometer at 37 °C, and were essentially as described by Bergmeyer (26). Enzyme activities were based on the amount of cellular protein, which was assayed according to Bradford (27) with bovine serum albumine as a standard.

NADH dehydrogenase was measured immediately after sonification at 340 nm, in the presence of 0.1 mM NADH and 0.01% cytochrome *c*. The rotenone-sensitive NADH dehydrogenase (NADH oxidase) activity was calculated by subtracting the rotenone-insensitive NADH dehydrogenase activity, as measured in the presence of 1 μM rotenone, from the total activity measured in the absence of rotenone. Succinate dehydrogenase was measured at 410 nm in the presence of 3 mM KCN, 1 mM potassium ferricyanide, and 5 mM succinate, according to Veeger *et al.* (28). α-Glycerophosphate dehydrogenase was assayed as succinate dehydrogenase, except that 5 mM α-glycerophosphate was used as a substrate; α-ketoglutarate dehydrogenase was measured at 340 nm, in the presence of 3 mM NAD^+, 50 μM coenzyme A, 2 mM KCN, and 1 mM α-ketoglutarate. Thiamine pyrophosphate was omitted from the assay, as this appeared to be without influence in the assay of homogenates. All enzyme activities mentioned above were assayed in 25 mM Tris/HCl (pH 7.4), 0.25 M sucrose, 2 mM EDTA, 10 mM K_2HPO_4, and 5 mM $MgCl_2$.

Glucose-6-phosphate dehydrogenase was assayed with 0.1 M triethanolamine buffer (pH 7.6), 0.9 mM $NADP^+$, and 10 mM glucose-6-phosphate; hexokinase with 0.1 M triethanolamine buffer (pH 7.6), 8 mM $MgCl_2$, 0.7 mM ATP, 0.9 mM $NADP^+$, 200 mM glucose, and 0.4 units/ml glucose-6-phosphate dehydrogenase; phosphofructokinase with 0.1 M phosphate buffer (pH 8.5), 1.4 mM $MgSO_4$, 0.7 mM phosphoenolpyruvate, 1.8 mM fructose 6-phosphate, 1.1 mM ATP, 0.4 mM NADH, 4 units/ml pyruvate kinase, and 9.6 units/ml lactate dehydrogenase; glyceraldehyde-3-phosphate dehydrogenase with 0.1 M phosphate buffer (pH 7.6), 3 mM NAD^+, and 0.45 mM glyceraldehyde 3-phosphate, in the absence or presence of 10 mM reduced glutathione; α-glycerophosphate dehydrogenase (cytoplasmic) with 0.1 M phosphate buffer (pH 7.0), 0.25 mM NADH, 1 mM EDTA, 1 mM KCN, and 10 mM dihydroxyacetone phosphate; pyruvate kinase with 0.1 M phosphate buffer (pH 7.0), 30 mM $MgSO_4$, 10 mM ADP, 0.25 mM NADH, 5 mM phosphoenolpyruvate, and 29 units/ml lactate dehydrogenase; lactate dehydrogenase with 0.1 M phosphate buffer (pH 7.0), 0.25 mM NADH, and 0.8 mM pyruvate; malate dehydrogenase with 0.1 M phosphate buffer (pH 7.0), 0.25 mM NADH, 1 mM KCN, and 1 mM oxaloacetate; glutathione reductase with 0.1 M phosphate buffer (pH 7.0), 0.3 mM NADPH, 1 mM EDTA, and 2 mM oxidized glutathione; lipoamide dehydrogenase with 0.1 M phosphate buffer (pH 7.0), 0.1 mM NADH, and 1 mM lipoamide; NADH diaphorase with 0.1 M phosphate buffer (pH 7.0), 0.25 mM NADH, 1 mM KCN, and 1 mM potassium ferricyanide.

Assays for Glucose, Pyruvate, Lactate, Amino Acids, and Ammonia in Culture Media—Net uptake or excretion of glucose, pyruvate, lactate, amino acids, and ammonia was calculated from changes in concentration as observed between fresh culture medium added to the cultures at day 3 and the same medium after 1 day of culturing time, *i.e.* at day 4. On days 3 and 4 parallel cultures were trypsinized and counted to correct for cell growth, as described by Rueckert and Mueller (5). A standard automated assay procedure was used for the determination of glucose and ammonia, an assay kit from Boehringer (Mannheim, F. R. G.) for that of pyruvate and L-lactate, and an aminoacid analyzer for that of the amino acids.

ATP Determination—Cellular ATP levels were assayed by bioluminometry, as described by Gille *et al.* (15).

RESULTS

Effect of Hyperoxia on Growth and Plating Efficiency—As shown in Fig. 1, hyperoxia caused inhibition of cell proliferation, which was most evident when applied at an earlier

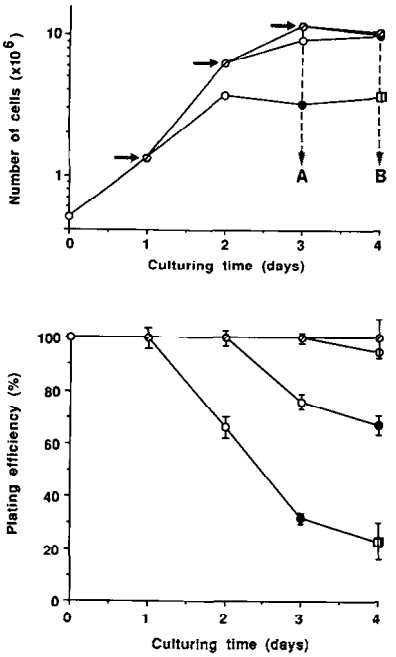

FIG. 1. **Proliferation (*upper graph*) and plating efficiency (*lower graph*) of CHO cells as a function of time under normoxia (∅) and the effect of shifting some of the cultures to hyperoxia for 1 (○), 2 (●), and 3 (□) days.** *Horizontal arrows* indicate times at which some cultures were shifted to hyperoxia. For studying cells that had been exposed to hyperoxia for 0, 1, and 2 days (*part A* in Figs. 2, 4–6) cultures were harvested at 3 days culturing time (*left vertical arrow*); to analyze cells that had experienced hyperoxia for 0, 1, 2, and 3 days (*part B* in Figs. 2, 4–6) cultures were harvested at 4 days culturing time (*right vertical arrow*). Results are means of eight independent experiments.

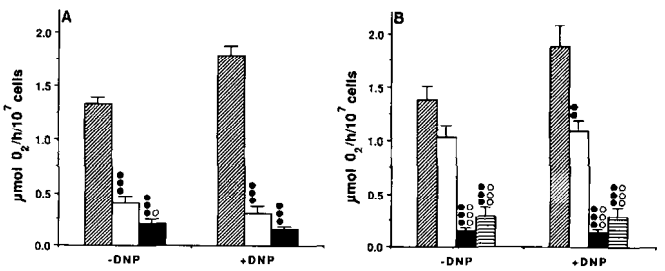

FIG. 2. **Hyperoxia-induced respiratory failure in CHO cells.** *A*, the effect of hyperoxia for 1 and 2 days on cellular oxygen consumption rates, as assessed at 3 days culturing time (*cf.* Fig. 1). *B*, the effect of hyperoxia for 1, 2, and 3 days, as observed at 4 days culturing time (*cf.* Fig. 1). *Bars* indicate from left to right: normoxic cells, cells kept at hyperoxia for 1, 2, and (*B* only) 3 days, respectively. Results are in means ± S.E.; $n = 8$ (*A*) or $n = 12$ (*B*). ○, ●, *, significantly different from 0, 1, and 2 days of hyperoxic exposure, respectively. Single, double, and triple symbols indicate probabilities of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively (Student's two-tailed *t* test).

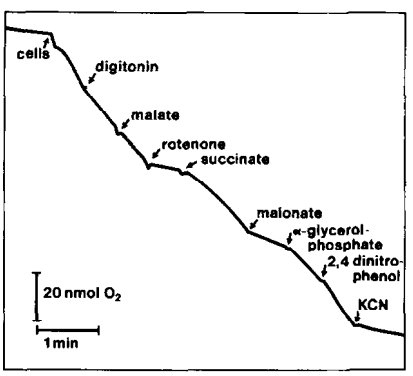

FIG. 3. **Polarographic recording of O_2 consumption by a suspension of intact CHO cells and the effect of permeabilization with digitonin and subsequent addition of substrates and inhibitors to measure the activities of NADH oxidase (complex I), succinate oxidase (complex II), and α-glycerophosphate oxidase complex, as described under "Materials and Methods."**

growth stage. When trypsinized and scored for plating efficiency the effect of hyperoxic exposure was more strikingly expressed. The plating efficiency was more severely reduced when cells were exposed to hyperoxia in an earlier growth phase. After 1 day of hyperoxia, plating efficiency appeared to be lowest at 2 days culturing time, intermediate at 3 days culturing time, and slightly reduced at 4 days culturing time. Two days of hyperoxia caused a reduction to approximately 30 and 70%, at 3 and 4 days culturing time, respectively. Finally 3 days of hyperoxia (at 4 days culturing time) reduced the plating efficiency to 23%.

Oxygen Consumption: Intact Cells—The effect of 1 and 2 days hyperoxic exposure on cellular oxygen consumption was examined at 3 days culturing time (Fig. 2*A*), while the effect of 1, 2, and 3 days hyperoxia was studied at 4 days culturing time (Fig. 2*B*). In both cases an inhibition of the cellular respiration rate was observed, the effect being expressed earlier at 3 days culturing time. In normoxic controls, coupling of oxidative phosphorylation was demonstrated by the induction of a 1.45-fold increase in the respiration rate upon addition of the uncoupling agent DNP. Remarkably, in all cases of hyperoxic exposure we failed to observe a stimulating effect of DNP, indicating a possible disturbance in the production of reducing equivalents or an inactivation of the ATP synthetase during hyperoxic exposure.

Oxygen Consumption: Digitonin-permeabilized Cells—Permeabilization of the cells by addition of digitonin appeared to have some effect on the rate of oxygen consumption (Fig. 3). Subsequent addition of malate did not alter the rate of respiration, which was taken to indicate that endogenous mitochondrial substrates were still available in sufficient quantities to drive respiration. The effects of successively added rotenone, succinate, malonate, α-glycerophosphate, DNP, and KCN were as expected (Fig. 3), allowing the quantification of key components of the respiratory chain, *i.e.* activities of the NADH, succinate, and α-glycerophosphate oxidase complexes.

As shown in Fig. 4, hyperoxia-induced respiratory failure appeared to be associated with a progressive reduction in NADH and succinate oxidase activities, whereas α-glycerophosphate oxidase activity remained largely unaffected. Moreover, the latter activity was still coupled to ATP synthesis, as shown by the stimulating effect of DNP. Therefore, a lack of reducing equivalents rather than a defect in the coupling of ATP synthesis to the electron transport chain may explain the observation that DNP failed to stimulate respiration in intact hyperoxia-exposed cells (*cf.* Fig. 2).

Spectrophotometric Analysis—In parallel experiments key enzymes of the respiratory chain and citric acid cycle were measured in cell homogenates using standard spectrophotometric assays. As shown in Fig. 5, a progressive inactivation of NADH dehydrogenase, succinate dehydrogenase, and α-ketoglutarate dehydrogenase activities was observed as a function of cellular exposure to hyperoxia. On the other hand, the activity of mitochondrial α-glycerophosphate dehydrogenase was unaffected, in agreement with the corresponding oxidase activity (*cf.* Fig. 4).

When comparing the data for normoxic control cells obtained by spectrophotometry with those obtained by respira-

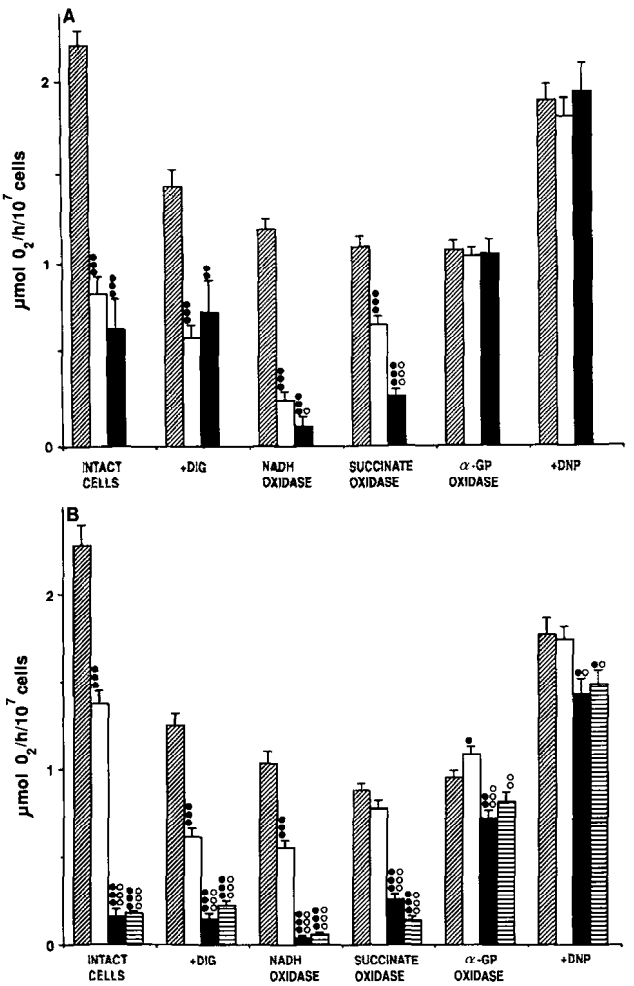

FIG. 4. **Key respiratory components in CHO cells exposed to hyperoxia.** Measurements were made at 3 (*A*) and 4 (*B*) days culturing time. Digitonin-treatment was used to measure the activities of NADH oxidase, succinate oxidase, and α-glycerophosphate oxidase, in the absence and presence of DNP (25 μM), as described under "Materials and Methods" and illustrated in Fig. 3. Results are in means ± S.E.; $n = 8$ (*A*) or $n = 12$ (*B*). For an explanation of the *bars* and *symbols*, see legend to Fig. 2. *DIG*, digitonin. Note that the respiration rate of intact cells is not directly comparable to that presented in Fig. 2, since different respiration buffers were used (see "Materials and Methods").

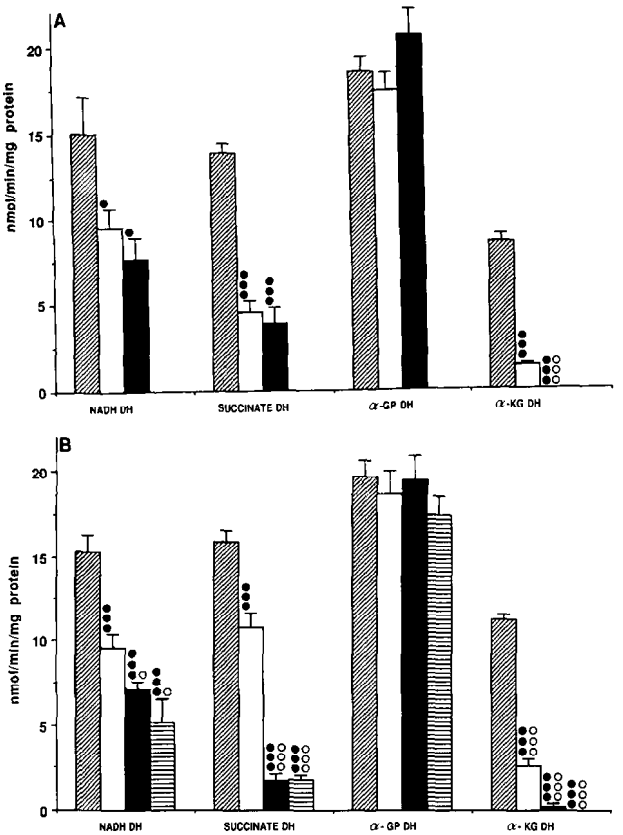

FIG. 5. **Activities of NADH, succinate, α-glycerophosphate, and α-ketoglutarate dehydrogenases in CHO cells exposed to hyperoxia, at 3 (*A*) and 4 (*B*) days culturing time.** Results are in means ± S.E.; $n = 8$ (*A*) or $n = 12$ (*B*). For an explanation of the *bars* and *symbols*, see legend to Fig. 2. *DH*, dehydrogenase; *α-GP*, α-glycerophosphate; *α-KG*, α-ketoglutarate.

tion measurements (Table I) the succinate and α-glycerophosphate dehydrogenase activities appeared to be identical, whereas a small discrepancy (20%) was observed between the activities of the NADH dehydrogenase complex. Thus, both techniques give roughly similar results, indicating that the measurement of oxygen consumption in digitonin-permeabilized cells can be used as a quick method to quantify these three mitochondrial enzyme activities on a per cell basis.

In view of the observed inactivation of mitochondrial metabolism and the impact this might have on energy metabolism, it was considered relevant to check the effect of hyperoxia on enzymes involved in glycolysis and in the maintenance of the NAD^+/NADH ratio. As shown in Table II, 1 day of hyperoxia did not induce any detectable change in these enzymes. However, after 2 days of hyperoxia, the activities of hexokinase, pyruvate kinase, and lactate dehydrogenase became elevated. After 3 days of hyperoxia, besides a still further enhancement of these three enzyme activities, an increase was also observed for the activities of phosphofructokinase, α-glycerophosphate dehydrogenase (cytoplasmic), and malate dehydrogenase. Remarkably, glyceraldehyde-3-phosphate dehydrogenase activity, an enzyme known to be selectively inactivated in hydrogen peroxide-exposed cells (23) was not inactivated by hyperoxia.

In view of the suspected importance of glutathione metabolism for cellular tolerance toward oxidative stress, glucose-6-phosphate dehydrogenase, a key enzyme of the pentose phosphate cycle, which is an important source of cellular NADPH, and glutathione reductase, were measured. Glutathione reductase activity did not change during hyperoxic exposure. On the other hand, glucose-6-phosphate dehydrogenase activity was slightly, but significantly, increased after 1 day of hyperoxia, followed by a further significant increase after 2 days of hyperoxia, whereafter no further increase was observed. This finding may suggest that the pentose phosphate shunt activity is enhanced under hyperoxic conditions, suggesting higher demands for NADPH and/or ribose sugar precursors for DNA and RNA synthesis.

As subunit *E*3 of the α-ketoglutarate dehydrogenase complex has been suggested to be oxygen-sensitive, this subunit was studied in more detail. Contrary to our expectation, hyperoxia did not affect lipoamide dehydrogenase activity. However, the activity of NADH diaphorase was significantly stimulated after 3 days of hyperoxia, indicating that hyperoxia somehow seems to affect subunit *E*3.

Changes in Glucose, Pyruvate, Lactate, Amino Acid, and Ammonia Concentrations in the Culture Medium—As an independent method to assess the relative contributions of glycolysis and oxidative metabolism to the energy metabolism of hyperoxia-exposed cells, in addition to enzyme activities, the concentrations of various metabolites were quantified in the culture medium of cells exposed to hyperoxia for 1, 2, and

TABLE I

Comparison of complex I, II, and α-glycerophosphate dehydrogenase activity as measured by polarographic and by enzymatic assays

For normoxic control cells spectrophotometric measurements were used to calculate the expected O_2 consumption rate and vice versa. Results are means ± S.E.; DH, dehydrogenase.

Enzymes	Spectrophotometry		Oxygen consumption	
	Measured ($n = 20$)	Calculated ($n = 12$)	Measured ($n = 12$)	Calculated ($n = 20$)
	nmol/min/mg protein		*μmol/h/10^7 cells*	
NADH (rotenone sensitive) DH	15.38 ± 0.79	19.06 ± 1.37[a]	1.03 ± 0.07	0.83 ± 0.04[a]
Succinate DH	16.05 ± 0.57	16.89 ± 0.96	0.91 ± 0.05	0.87 ± 0.03
α-Glycerophosphate DH	18.02 ± 1.06	17.57 ± 0.72	0.95 ± 0.04	0.98 ± 0.06

[a] Significantly different from the measured value ($p < 0.05$).

TABLE II

Key enzymes of glycolysis and several other metabolic pathways during exposure to hyperoxia

Enzyme activities are expressed as nmol substrate·min^{-1}·mg $protein^{-1}$ (means ± S.E.; $n = 8$); DH, dehydrogenase; cyt, cytoplasmic. ^{*,0}†, significantly different from 0, 1, and 2 days of hyperoxic exposure, respectively. Single, double, and triple symbols indicate probabilities of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively (Student's two-tailed t test).

Enzymes	Time of hyperoxia (days)			
	0	1	2	3
Hexokinase	24.3 ± 1.6	23.2 ± 1.3	28.2 ± 1.1^{0}	34.5 ± 2.4^{**,00,†}
Phosphofructokinase	77.1 ± 2.5	73.5 ± 4.2	81.4 ± 2.0	101.3 ± 7.5^{**,00,†}
Glyceraldehyde-3-P DH[a]	1261 ± 103	1155 ± 85	1441 ± 77	1644 ± 167
α-Glycerophosphate DH (cyt)	1460 ± 110	1444 ± 88	1504 ± 99	1845 ± 143^{0}
Pyruvate kinase	2797 ± 119	2637 ± 95	3085 ± 137^{0}	3579 ± 110^{***,000}
Lactate dehydrogenase	1929 ± 163	1865 ± 112	2407 ± 177^{0}	2842 ± 143^{***,000}
Malate dehydrogenase	1122 ± 88	1074 ± 32	1188 ± 103	1631 ± 165^{*,00,†}
Glucose-6-phosphate DH	53.9 ± 0.8	59.2 ± 1.6*	91.2 ± 4.6^{***,000}	81.6 ± 4.5^{***,000}
Glutathione reductase	58.3 ± 2.7	53.0 ± 2.0	62.9 ± 3.2	56.1 ± 7.1
Lipoamide dehydrogenase	65.1 ± 2.8	57.6 ± 2.1	65.6 ± 4.0	65.1 ± 3.2
NADH diaphorase	1665 ± 131	1587 ± 110	1779 ± 94	3362 ± 323^{***,000,†}

[a] Measured in the presence of 10 mM GSH; without GSH in the assay mixture the overall levels of activity were reduced, but no effect of cellular exposure to hyperoxia was observed.

3 days. Glucose, pyruvate, alanine, and lactate were measured as markers for glycolytic activity, while for oxidative metabolism glutamine, glutamate, and aspartate were quantified (19, 21, 29). For the sake of completeness the other amino acids were assayed as well.

As shown in Table III, glucose consumption and lactate production increased significantly during the second and third days of hyperoxic exposure, whereas pyruvate concentrations did not change. On the other hand, glutamine consumption decreased from the first day of hyperoxia and even changed into a net production during the second day, followed by a slight consumption during the third day of hyperoxia (Table III). A similar pattern was found for aspartate, arginine, and histidine, suggesting a possible interrelationship between these changes. Cellular utilization of glutamate ceased during the first day of hyperoxia, after which this amino acid started to accumulate in the culture medium; ammonia production tended to decrease as a function of hyperoxic exposure time. Alanine production became significantly reduced during the second and third day of hyperoxia, while glycine production and serine consumption both showed a tendency to increase during hyperoxic exposure. Hyperoxia appeared to have no significant effect on the amino acids asparagine, valine, leucine, phenylalanine, lysine, proline, and threonine. Data for the other amino acids, *i.e.* isoleucine, tryptophan, methionine, cysteine, and tyrosine, are not shown, as differences were below the detection level.

ATP—In view of the observed dramatic changes induced by hyperoxia in the energy metabolism of CHO cells, cellular ATP levels were measured. As shown in Fig. 6, at both 3 and 4 days culturing time, ATP levels were significantly decreased (30–40%) after 2 days of hyperoxia (Fig. 6*A*). At 4 days culturing time (Fig. 6*B*) a further depletion of ATP (60%) was observed after 3 days of hyperoxia.

DISCUSSION

In this paper we document that CHO cells under normobaric hyperoxia (98% O_2) suffer from a progressive respiratory failure. We have shown that this loss of respiratory activity is associated with a selective inactivation of at least three SH-group-containing flavoprotein complexes, *i.e.* the NADH dehydrogenase, succinate dehydrogenase, and most strikingly the α-ketoglutarate dehydrogenase complex. Since these enzyme complexes are localized at or near the inner mitochondrial membrane, it is tempting to assume that their inactivation under hyperoxia is mediated by free radicals, generated by the ubiquinone-cytochrome *c* reductase complex (31, 32). As the earliest symptom an apparent loss of susceptibility to the uncoupler DNP is observed, which should be interpreted as being due to a lack of NADH reducing equivalents rather than to true uncoupling, since in digitonin-permeabilized cells respiration driven by α-glycerophosphate via α-glycerophosphate oxidase is still responsive to DNP (Fig. 4). This indicates that ubiquinone cytochrome *c*-reductase (complex III), cytochrome *c*-oxidase (complex IV), and ATP-synthetase (complex V) are hardly if at all affected by hyperoxia. Consequently, hyperoxia-exposed cells possess an active ATP synthetase but are unable to produce ATP by oxidative phosphorylation as a result of the combined inactivations of the above-mentioned enzymes. These changes precede the loss of

TABLE III

Effect of hyperoxia on cellular consumption/production of glucose, pyruvate, lactate, amino acids, and ammonia

Concentration changes are in nmol·h^{-1}·10^{-7} cells (means ± S.D.; n = 4). Positive sign is production, negative sign is consumption. For symbols, see Table II.

Compound	Time of hyperoxia (days)			
	0	1	2	3
Glucose	−2,675 ± 238	−2,078 ± 239*	−4,680 ± 391***,000	−6,330 ± 667***,000,††
Pyruvate	−71 ± 10	−71 ± 24	−66 ± 34	−48 ± 23
Lactate	3,888 ± 392	3,563 ± 221	8,153 ± 770***,000	10,590 ± 440***,000,††
Glutamine	−430 ± 71	−314 ± 23*	58 ± 68***,000	−96 ± 57***,000,†
Glutamic acid	−33 ± 15	8 ± 6***	40 ± 24**,0	102 ± 57**,0
Alanine	274 ± 29	245 ± 29	158 ± 28**,00	108 ± 16***,000,†
Asparagine	−74 ± 11	−68 ± 3	−71 ± 29	−72 ± 24
Aspartic acid	−33 ± 7	−16 ± 4**	32 ± 24**,00	−21 ± 10††
Serine	−79 ± 20	−85 ± 6	−112 ± 9*,00	−147 ± 19**,000,†
Glycine	125 ± 8	57 ± 5***	113 ± 33^{0}	220 ± 27***,000,††
Arginine	−118 ± 42	−67 ± 26	3 ± 53*	−26 ± 40
Valine	−36 ± 4	−37 ± 2	−37 ± 6	−24 ± 3**,00,††
Leucine	−74 ± 7	−65 ± 8	−66 ± 3	−69 ± 11
Phenylalanine	−19 ± 2	−19 ± 2	−15 ± 3	−8 ± 4**,00,†
Histidine	−19 ± 11	−1 ± 2*	9 ± 12*	−17 ± 11^{0},†
Lysine	−47 ± 13	−39 ± 7	−23 ± 23	−23 ± 66
Proline	−33 ± 7	−20 ± 11	−21 ± 8	−2 ± 7***,0,†
Threonine	−36 ± 4	−35 ± 1	−34 ± 9	−29 ± 11
Ammonia	721 ± 123	535 ± 44	408 ± 71	356 ± 106

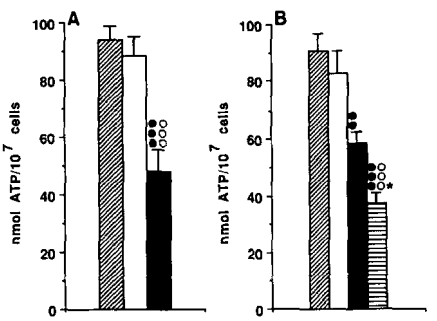

FIG. 6. **ATP levels in CHO cells exposed to hyperoxia at 3 (*A*) and 4 (*B*) days culturing time.** Results are in means ± S.E.; n = 16 (*A*) or n = 20 (*B*). For an explanation of the *bars* and *symbols* see legend to Fig. 2.

clonogenicity under hyperoxic growth conditions, which implies that these events may reflect primary target processes in hyperoxic intoxication.

As oxidative metabolism in cell culture is highly dependent on glutamine and/or glutamate consumption (19–21), the observed decrease in the total consumption of glutamine and glutamate during hyperoxic exposure was as expected. Moreover, it should lead to an enhanced glycolysis, as was confirmed by the evident increase in glucose consumption and lactate production. Thus, paradoxically, in hyperoxia-exposed CHO cells, energy metabolism is shifted to glycolysis. Increased glycolysis under hyperoxia has been repeatedly described previously, *e.g.* by Rueckert and Mueller (5), Allen and Rasmussen (24), Balin *et al.* (7, 8), and Van der Valk *et al.* (13), however, without clearly linking this phenomenon to respiratory failure.

The enhanced glycolysis may be due to enhanced levels of glycolytic regulatory enzymes (33) or to activation of allosteric enzymes. Both mechanisms appeared to be operative, because a clear 1.2–1.7-fold enhancement in key regulatory enzymes, as well as a good correlation with the depletion of ATP, an allosteric inhibitor of phosphofructokinase (34, 35), were observed. An inactivation of glycolysis, as reported for oxidative stress induced by hydrogen peroxide, by selective damage to glyceraldehyde-3-phosphate dehydrogenase (23), was not observed in the case of hyperoxia. This suggests, that the toxicity of normobaric hyperoxia is based on a different mechanism from that of hydrogen peroxide (15, 23).

A 3-fold enhancement of the glycolytic flux during hyperoxia could not prevent a decrease in ATP levels after hyperoxic exposure. The decrease was not due to exhaustion of glucose from the culture medium, as abundant glucose (>2.5 mM) was left in the culture fluid at the end of the experiment. On the other hand, a mutant with a respiratory deficiency, caused by an inactive NADH dehydrogenase, and with half-maximal ATP levels (36), could grow almost as fast as the wild-type cell line, while showing a two times enhanced glycolytic flux (19, 37). However, this mutant, kindly provided by Prof. I. E. Scheffler, University of California, San Diego, was unable to grow under hyperoxia.[2] Hence, the importance of an inactivation of the α-ketoglutarate dehydrogenase complex should not be underestimated. A deficiency in man of this enzyme, particularly lipoamide dehydrogenase, leads to death in early infancy (38, 39), while a deficiency in NADH dehydrogenase is not lethal, but leads to mitochondrial myopathies (40–42). In view of this, the activity of lipoamide dehydrogenase was also examined under hyperoxic stress, but appeared to be insensitive (Table II). On the other hand, an artificial activity of this enzyme (NADH diaphorase) was increased after hyperoxic exposure, indicating that the *E*3 subunit is somehow affected by hyperoxia. Another important target in oxygen toxicity might be subunit *E*2, the dihydrolipoamide acyltransferase, to which a lipoic acid cofactor is attached via a lysine moiety (22). Further work is required to elucidate this point.

NADPH can be synthesized in the pentose phosphate cycle to restore GSH from GSSG (1, 24, 43), an important process in the defense against oxidative stress. This could explain the enhancement of glucose-6-phosphate dehydrogenase activity under hyperoxic growth conditions. Another possibility is, that with the ribose sugars nucleotides are synthesized, which may be needed for an increased rate of DNA excision repair (11). Remarkably, glutathione reductase was unaffected by hyperoxia, although this enzyme contains essential sulfhydryl groups. In this respect, it is similar to the lipoamide and

[2] W. G. E. J. Schoonen, unpublished data.

glyceraldehyde-3-phosphate dehydrogenases, which were also resistant to hyperoxia.

The present results indicate that the process leading to hyperoxia-induced clonogenic death of CHO cells is likely to be initiated by the rapid and selective inactivation of three mitochondrial SH group-containing dehydrogenases, which causes the electron transport chain to become deprived of reducing equivalents. The resulting ATP depletion induces an increase in glucose metabolism, partly by activating key allosteric enzymes. This, however, does not appear to be sufficient to prevent further depletion of ATP, so that loss of viability has become inevitable. This particular intoxication mechanism shows a striking resemblance with that described for the killing of neoplastic cells by cytotoxic macrophages (17). This killing was caused by respiratory failure and nearly complete inhibition of complex I and II, while α-glycerophosphate dehydrogenase and the complexes III, IV, and V were hardly or not affected. For the maintainance of cell viability, these cells also required glucose (44). Recently, it has been reported that tumor necrosis factor-α, a cytokine secreted by macrophages upon activation (45), is also able to induce a specific inhibition of the bioenergetic machinery (46), while oxygen radicals have been implicated in the mechanism of action of tumor necrosis factor-α (47). Thus, selective inactivation of certain mitochondrial functions may be part of a more general mechanism of oxidative cell killing.

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