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EFFECT OF HYPOXIA-REOXYGENATION ON PEROXISOMAL FUNCTIONS IN CULTURED HUMAN SKIN FIBROBLASTS FROM CONTROL AND ZELLWEGER SYNDROME PATIENTS

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To delineate the role of peroxisomes in the pathophysiology of hypoxia-reoxygenation we examined the functions of peroxisomes and mitochondria in cultured skin fibroblasts from controls and from patients with cells lacking peroxisomes (Zellweger cells). The loss of peroxisomal functions (lignoceric acid oxidation and dihydroxyacetonephosphate acyltransferase [DHAP-AT] activities) in control cells following hypoxia and hypoxia followed by reoxygenation, suggests that peroxisomes are sensitive to oxidative injury. The sensitivity of peroxisomes to oxidative stress was compared to that of mitochondria by examining the oxidation of palmitic acid (a function of both mitochondria and peroxisomes) in control and Zellweger cell lines, following hypoxia-reoxygenation. The greater loss of activity of palmitic acid oxidation observed in control cells as compared to that seen in Zellweger cells suggests that the peroxisomal β-oxidation system is relatively more labile to hypoxia- reoxygenation induced oxidative stress. This data clearly demonstrates the difference in the response of mitochondria and peroxisomes to oxidative stress.

KEY WORDS: Hypoxia, reoxygenation, fibroblasts, peroxisomes, mitochondria, β-oxidation.

INTRODUCTION

Oxygen derived free radicals and reactive oxygen species are normal metabolic by-products of cellular metabolism and are detoxified by various cellular enzymatic and non-enzymatic systems. Under normal conditions these antioxidant defense mechanisms are adequate. Hypoxia followed by reoxygenation generates increased amounts of free radicals[1,2] which are known to damage various cellular constituents such as proteins, lipids, nucleic acids and carbohydrates thus adversely affecting the normal function of the cells.

Peroxisomes have been identified in all tissues studied thus far, with the exception of red blood cell.[3] In the past 25 years more than 60 enzymes associated with important metabolic functions (including the synthesis of plasmalogens and bile acids, oxidation of very long chain fatty acids, mono-and polyunsaturated fatty acids and prostaglandins) have been identified in peroxisomes.[3] Since both enzymes producing the reactive oxygen species (e.g. Oxidases,[3,4] Cytochrome P-450,[5] Cytochrome

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b5 reductase and Cytochrome P-450 reductase[6]) and the antioxidant enzyme systems (CuZnSOD,[7,8] MnSOD,[9] GPx[9] and catalase) are found in peroxisomes, one can assume that peroxisomes play a significant role in the cellular antioxidant defense mechanism. Earlier our laboratory demonstrated that exposure of kidney to ischemia-reperfusion insult leads to changes in the structure and function of peroxisomes.[10,11]

In control cells the peroxisomal oxygen-utilization varies from 5–20% of the total cellular oxygen consumed which is converted to H_2O_2 and probably to small amounts of superoxide.[12] This makes the peroxisomal detoxification of reactive oxygen species essential. Similar to other organelles peroxisomes also experience oxidative stress during hypoxia-reoxygenation. The availability of fibroblast cell lines which lack functional peroxisomes provided an ideal model to study the relative lability of different organelles under oxidative stress. Fibroblast cell lines from patients suffering from the Cerebro-Hepato-Renal-Syndrome (Zellweger Syndrome) lack functional peroxisomes. Although catalase is a peroxisomal enzyme in control cells, in Zellweger cells due to the absence of peroxisomes catalase is present mostly in the cytoplasm. We exposed both, control and Zellweger cells to hypoxia and reoxygenation to monitor the changes of peroxisomal and mitochondrial enzyme systems and to show the lability of mitochondrial vs. peroxisomal functions under conditions of oxidative stress.

MATERIALS AND METHODS

Cell lines (fibroblasts) and tissue culture conditions Zellweger cell lines (GM 00228 and GM 04340) were obtained from the NIGMS Human Genetic Mutant Cell Repository. Control cell lines were derived from healthy subjects. All cells were used in passages 8–14. Fibroblasts in 100 mm tissue culture dishes were grown to confluence in Dulbecco's Modified Eagle medium (DMEM, Gibco) supplemented with 15% bovine calf serum (BCS, Hyclone) and gentamycin (50 μg/ml) under isobaric conditions (5% CO_2, air). Cells were harvested by trypsinization. After trypsinization cells were spun down, resuspended in tissue culture media and kept at 37°C in a shaking waterbath for 1 hour to restore cellular membrane proteins damaged by trypsinization prior to assays or used immediately without incubation at 37°C for the assays after hypoxia without reoxygenation.

Induction of hypoxia and reoxygenation Zellweger and control fibroblast monolayers were subjected to hypoxia in a GasPak anaerobic chamber (BBL Microbiology Systems, Becton Dickinson, Cockeysville, MD) at 37°C for up to 4 hours. This system chemically removes oxygen, resulting in an atmosphere of less than 0.4% oxygen and 5% carbon dioxide in 100 min. Cells were inserted into the chamber immediately before reducing the oxygen concentration. The concentration of soluble oxygen was determined with a dissolved oxygen meter (Omega engineering, Stamford, Connecticut, Model PHH-71). Control and Zellweger cells seeded and harvested in parallel but not subjected to hypoxia served as the non-hypoxic, non-reoxygenated controls (100% values).

Protein was estimated by the method of Bradford using Bovine Serum Albumin as a standard.[13]

Viability of cells Cells were stained with Trypan Blue and the percentage of living cells was calculated.

Catalase Cytosolic and membrane bound catalase were distinguished by digitonin treatment of trypsinized cultured skin fibroblasts[14] and catalase enzyme activity was measured as described previously.[15]

Assay for dihydroxyacetone phosphate acyltransferase activity (DHAP-AT) The enzyme activity of DHAP-AT was measured according to the procedure described previously.[16] Briefly, [U-^{14}C]DHAP (the substrate for DHAP-AT) was synthesized from glycerol-3-phosphate (G3P). The reaction mixture of 1 ml contained 10 μCi of [U-^{14}C]G3P (27 mCi/mmol), 0.6 mM G3P, 5 mM pyruvate, 1 mM NAD^+, lactate dehydrogenase (10 units), α-glycerol-3-phosphate dehydrogenase (10 units), and 50 mM triethanolamine buffer, pH 7.6. After 60 min incubation at room temperature, the reaction was stopped by the addition of chloroform, and the upper phase contained [U-^{14}C]DHAP.[16]

For the assay of DHAP-AT, a reaction mixture containing 0.1 mM [U-^{14}C]DHAP, 8 mM $MgCl_2$, 8 mM sodium fluoride, 0.4 mg albumin, 0.15 mM palmitoyl-CoA, and 75 mM acetate buffer, pH 5.4 in total volume 0.12 ml, was incubated at 37°C for 1 h. The reaction was stopped with 0.45 ml of chloroform: methanol (2:1) followed by the addition of 150 μl chloroform and 150 μl 2 M KCl/0.2 M H_3PO_4. After centrifugation and removal of the upper phase, 200 μl of the lower phase was applied to filter papers (Whatman #3), which were dried at room temperature and then washed with trichloroacetic acid. The filter papers were dried again, and the radioactivity was counted.

Oxidation of [1-^{14}C]Lignoceric Acid and [1-^{14}C]Palmitic Acid The fatty acid oxidation assays were performed on cell suspensions of trypsinized, unbroken fibroblasts from control and Zellweger cell lines under isotonic conditions.

[1-^{14}C]lignoceric acid (54 mCi/mmol, 24 × 10^6 DPM) or [1-^{14}C]palmitic acid (55 mCi/mmol, 24 × 10^6 DPM) were suspended in 4 ml of a solution of α-cyclodextrin (20 mg/ml) in isotonic buffer (0.25 M sucrose, 3 mM imidazole, pH = 7.2, 1 mM EDTA, 0.1% ethanol) by sonication. The reaction was started by the addition of [1-^{14}C]lignoceric acid or [1-^{14}C]palmitic acid (0.15 × 10^6 DPM) to fibroblasts (100–150 μg protein) suspended in 200 μl isotonic buffer. The tubes were incubated for 30 minutes or 1 hr at 37°C in a shaking waterbath. The reaction was stopped by the addition of 0.625 ml of 1 M KOH in methanol followed by incubation at 60°C in a shaking waterbath for 1 hour. The amount of radioactivity in the upper phase of the Folch partition represents the rate of oxidation of lignoceric or palmitic acid.[15,17]

RESULTS AND DISCUSSION

Human skin fibroblasts derived from control subjects and Zellweger syndrome patients were subjected to hypoxia in a GasPak anaerobic chamber for two to four hours and returned to normal oxygen tension for 1 to 48 hours. To verify that cells in the hypoxic and reoxygenated dishes were not lost due to detachment following trypsinization the number of cells was determined in all samples and compared with control counts. Also the number of viable cells was determined prior to performing the biochemical assays. Both the absolute cell numbers and the percentage of viable cells in controls (95 ± 3%) and hypoxic and/or reoxygenated (93 ± 5%) dishes did not show a significant difference under these experimental conditions.

The activities of peroxisomal enzymes (catalase, dihydroxyacetonephosphate

TABLE 1
Specific enzymatic activities of untreated control and Zellweger cells

Zellweger syndrome (CHRS)	
Catalase:	7.59 ± 0.41 mU/mg protein
Particle bound catalase:	9 ● 4% of total catalase
Oxidation of lignoceric acid:	5.2 ± 1.9 pmol/hr/mg protein
DHAP-AT	35.7 ± 9.9 pmol/hr/mg protein
Oxidation of palmitic acid:	511 ± 22 pmol/hr/mg protein
Control	
Catalase:	4.45 ± 0.29 mU/mg protein
Particle bound catalase:	83 ± 7% of total catalase
Oxidation of lignoceric acid:	117 ± 9 pmol/hr/mg protein
DHAP-AT	717 ± 49 pmol/hr/mg protein
Oxidation of palmitic acid:	553 ± 32 pmol/hr/mg protein

Data are expressed as the mean value of 4–6 experiments ± SD.

acyltransferase [DHAP-AT] and the enzyme system for the oxidation of lignoceric acid) are shown in Table 1. DHAP-AT, the first enzyme in the synthesis of plasmalogens[18] and alkyl-DHAP synthetase are localized in peroxisomes, while the enzymes for the subsequent steps are localized in the endoplasmic reticulum.[3,18] The activity of DHAP-AT was only 5% of the control values in the Zellweger fibroblasts (Table 1) which is in agreement with previous studies.[19] This lack of peroxisomal enzymes results in lower levels of plasmalogens in Zellweger cells. Plasmalogens are essential constituents of membranes and are also thought to function as free radical scavengers for the reactive oxygen species.[20,21] Due to the lack of peroxisomes the rate of β-oxidation of lignoceric acid (a peroxisomal function) was 5% of control.[22] In control fibroblasts approximately 80% of the catalase activity is present in peroxisomes whereas in Zellweger cells less than 10% of catalase is membrane associated (Table 1). Surprisingly the overall specific activity of catalase in Zellweger cells is 1.7 times higher than in control cells. (Table 1).

Figure 1 shows the specific activities of DHAP-AT and the enzyme system for the β-oxidation of lignoceric acid in control cells in response to hypoxia and reoxygenation. The activities of DHAP-AT and lignoceric acid oxidation showed a significant decrease during hypoxia, followed by a second drop in activity during the first hour of reoxygenation and recovery of activity within 48 hours of reoxygenation, suggesting that cells exposed to these conditions of hypoxia and reoxygenation were not irreversibly damaged (Figure 1). However, these results do clearly demonstrate that during reoxygenation the peroxisomal functions (synthesis of plasmalogens and oxidation of very long chain fatty acids) were significantly compromised (Figure 1). We did not examine the effect of hypoxia-reoxygenation on the activity of DHAP-AT and lignoceric acid oxidation in Zellweger cells because these activities were only 5% of the control values. This makes it impossible to detect significant changes in these activities in Zellweger fibroblasts.

Since significant production and detoxification of reactive oxygen species occurs within the peroxisomal membrane boundaries,[3,23] peroxisomes must contain efficient systems for their detoxification to protect the peroxisomal enzymatic functions from oxidative damage. Therefore, we examined the effect of hypoxia-reoxygenation on the activity of catalase in control and Zellweger cells. Catalase activity was not significantly effected during hypoxia and reoxygenation in control cells (Figure 2).

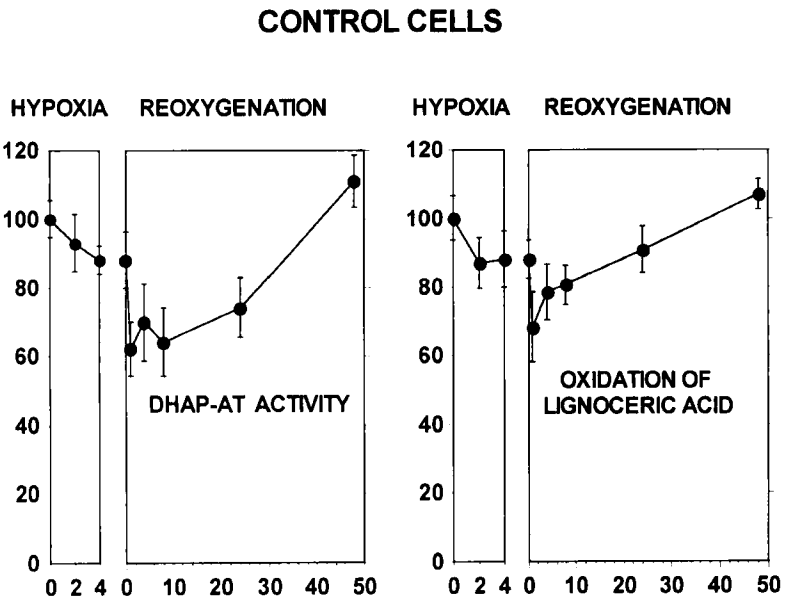

FIGURE 1 Control fibroblasts were subjected to hypoxia and reoxygenation. Activities given (% activity ± % standard deviation of 3 experiments) are expressed as percent of the specific activity of cells from the same cell line cultivated in parallel but not subjected to hypoxia or reoxygenation. Activities were determined in fibroblast homogenates (DHAP-AT) or intact cells (oxidation of lignoceric acid). Y-Axis: Percent activity as compared to cells not subjected to hypoxia and reoxygenation. X-axis: Time of hypoxia/reoxygenation in hours. For specific activities see Table 1.

On the other hand Zellweger cells experienced a small but significant loss of catalase activity during 4 hours of hypoxia and 8 hours of reoxygenation, however this normalized within 48 hours of reoxygenation (Figure 2).

The response of mitochondria and peroxisomes to oxidative stress was studied with respect to palmitic acid oxidation (Figure 3). Unlike the β-oxidation of lignoceric acid, which is a peroxisomal function,[17] palmitic acid is β-oxidized in both mitochondria and peroxisomes. We compared the rate of oxidation of palmitic acid in control cells (cells which contain systems for both peroxisomal and mitochondrial β-oxidation of palmitic acid) with Zellweger fibroblasts (cells which can only perform mitochondrial β-oxidation of palmitic acid) at 2–4 hours of hypoxia and 1–48 hours of reoxygenation following hypoxia (Figure 3). The activity of palmitic acid oxidation was significantly decreased during hypoxia in control cells (73% of untreated controls) and further decreased during the first hour of reoxygenation (60% of untreated control cells, Figure 3). A relatively greater loss of activity for the β-oxidation of palmitic acid was observed in control cells as compared to that seen in Zellweger cells (Figure 3). The normalization of palmitic acid oxidation in both control and Zellweger cells following prolonged reoxygenation (48 hrs) suggests that these cells were not irreversibly damaged under these experimental conditions. The greater loss of activity of oxidation of palmitic acid in control cells as compared to Zellweger cells suggests that the mitochondrial fatty acid β-oxidation system is better protected against inactivation by oxidative stress than the peroxisomal β-oxidation system. Under stress conditions, up to 20% of the cellular oxygen is consumed by

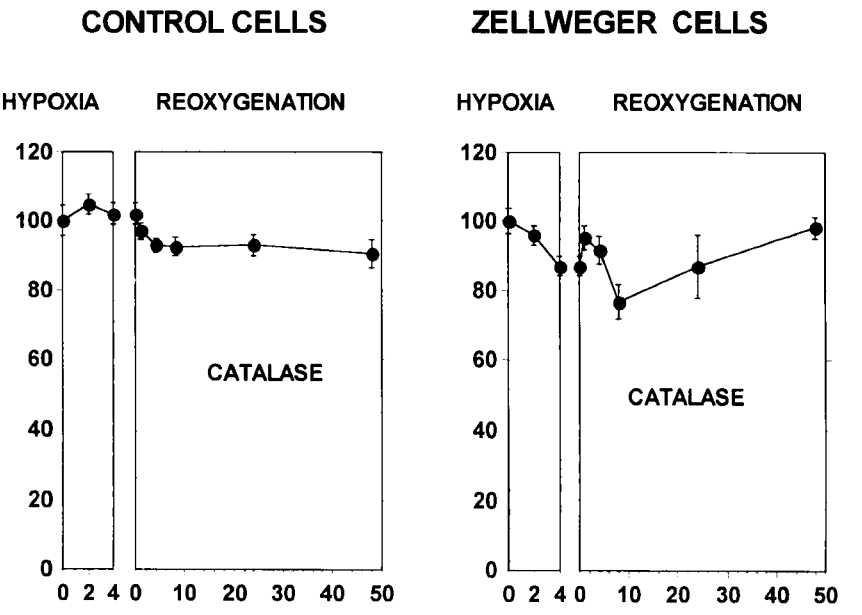

FIGURE 2 Control and Zellweger fibroblasts were subjected to hypoxia and reoxygenation. Activities given (% activity ± % standard deviation of 3 experiments) are expressed as percent of specific activity of cells from the same cell line cultivated in parallel but not subjected to hypoxia or reoxygenation. Catalase activities were determined in fibroblast homogenates. Y-Axis: Percent activity as compared to cells not subjected to hypoxia and reoxygenation. X-axis: Time of hypoxia/reoxygenation in hours. For specific activities see Table 1.

peroxisomes and converted to hydrogen peroxide and superoxide which may overwhelm the peroxisomal detoxification system. Since significant amounts of reactive oxygen species can be produced within the peroxisomes, enzyme systems vital to the cellular function are at risk of being inactivated or destroyed.

Due to oxidative stress, inactivation of plasmalogen synthesis (Figure 1), together with degradation of membrane phospholipids,[1,2] can lead to lowered levels of plasmalogens in organellar membranes reducing their fluidity and capacity to scavenge oxygen radicals.[20,21,24,25] The peroxisomal β-oxidation system is associated with not only the oxidation of long and very long chain saturated fatty acids but also with the oxidation of dicarboxylic acids, unsaturated fatty acids, eicosanoids and the oxidation of prostaglandins.[3,26] Arachidonic acid is released from phospholipids under conditions of oxidative stress resulting in higher levels of arachidonic acid and prostaglandins.[27] Since the peroxisomal β-oxidation system is damaged under these stress conditions, one can assume that these already enhanced levels of fatty acids including arachidonic acid and its metabolites can not be handled adequately by a damaged peroxisomal β-oxidation system thus leading to secondary damage of other enzyme systems and organelles.[28]

In summary, on exposure to hypoxia-reoxygenation, the peroxisomal β-oxidation system is more susceptible to damage than the mitochondrial system. This data suggests that during oxidative stress, changes in compartmentalized antioxidant defense mechanisms play a pivotal role in the overall cellular antioxidant defense systems.

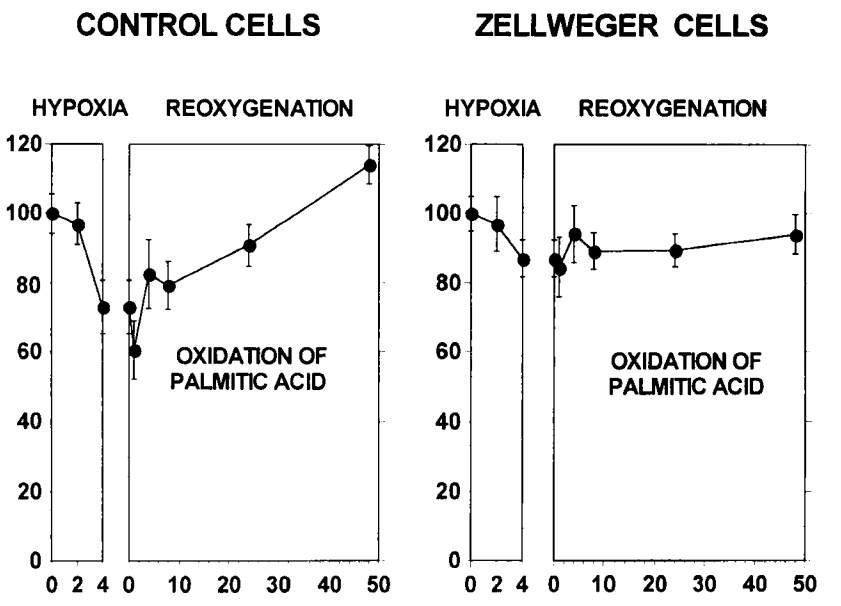

FIGURE 3 Control and Zellweger fibroblasts were subjected to hypoxia and reoxygenation. Activities given (% activity ± % standard deviation of 3 experiments) are expressed as percent of the specific of cells from the same cell line cultivated in parallel but not subjected to hypoxia or reoxygenation. Activities were determined in intact cells (oxidation of palmitic acid). Y-Axis: Percent activity as compared to cells not subjected to hypoxia and reoxygenation. X-axis: Time of hypoxia/reoxygenation in hours. For specific activities see Table 1.

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