Plasma Membrane Cholesterol: A Possible Barrier to Intracellular Oxygen in Normal and Mutant CHO Cells Defective in Cholesterol Metabolism †

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Plasma Membrane Cholesterol: A Possible Barrier to Intracellular Oxygen in Normal and Mutant CHO Cells Defective in Cholesterol Metabolism†

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ABSTRACT: The effect of the cholesterol content of the plasma membrane on the intracellular concentration of oxygen in Chinese hamster ovary (CHO) cells and their mutants was investigated by EPR oximetry. Total and free cholesterol content was significantly higher in 25 RA CHO cells as compared to wild-type and M 19 CHO cells, with most of the free cholesterol in normal and mutant CHO cells located in the plasma membrane. The plasma membrane cholesterol content also was altered by various biochemical means, and the effect on the oxygen gradient was studied. Comparing the three cell lines, the gradient was larger with increased content of cholesterol in the plasma cell membrane. This result also is supported by an additional increase in the oxygen gradients with the incorporation of additional cholesterol in the plasma membrane and a decrease in the oxygen gradient when the cholesterol was depleted from the plasma membrane. The results indicate that the concentration of cholesterol in the plasma membrane can be an important factor for the magnitude of the oxygen gradient observed across the cell membrane.

The concentration of oxygen within cells is an important parameter in many physiological and pathological processes. A number of methods have been developed to measure it, using polarographic electrodes, mass spectrometry, NMR, and fluorescence polarization (*1*−*4*). Values of the oxygen concentration difference (gradient) in cells reported in the literature vary widely: from small, lower than 1 μM (*5*, *6*), to large, as great as 40 μM or more (*7*−*26*). While the number of reports on the occurrence of oxygen gradients has continued to increase, there still is no consensus on the validity of the concept of the occurrence of oxygen gradients in cells and the mechanism involved. One of the primary reasons for the discrepancy is the technical difficulty of making valid measurements of intracellular oxygen concentration under physiological conditions. Consequently, our efforts during the last several years have included improving the methodology of EPR[1] oximetry to provide more precise and accurate data, examining systems in which oxygen gradients may occur, and probing the mechanisms by which the oxygen gradients occur (*10*−*12*, *17*−*26*). We have suggested that the plasma membrane could be one of the factors responsible for restricted diffusion of oxygen in cells (*12*, *20*).

In the plasma membrane, cholesterol is a principal nonpolar hydrophobic lipid, and phospholipids and glycolipids are the principal polar hydrophilic lipids. While the role of cholesterol in the plasma membrane is a long-standing puzzle, recent studies suggest that it can affect oxygen transport. It has been reported that the plasma membrane cholesterol influences oxygen release, transport, and availability in red blood cells (*27*). Dumas et al. (*28*) investigated oxygen diffusion in membranes of monolayer human endothelial cells using fluorescent techniques, and the results indicated a decrease in oxygen diffusion rates as the cholesterol concentration was increased in the medium culture. It has been demonstrated that cholesterol can decrease the permeability of lipid bilayer membranes to oxygen by severalfold (*29*−*33*). Cholesterol also decreased nitric oxide transport (*34*, *35*) by decreasing the permeability in the RBC by 2000-fold more than previously thought (*36*) and also can affect the transport of larger hydrophobic molecules (*30*).

Various CHO cell mutants with defined mutations in the cholesterol metabolism are available (*37*). To elucidate the possible role of plasma membrane cholesterol in regulating the intracellular concentration of oxygen, we investigated the extra- and intracellular oxygen concentration in CHO cells and their mutants that have different plasma membrane cholesterol concentrations: wild-type, 25 RA, and M 19 CHO cells. Genes encoding the low-density lipoprotein receptor and various enzymes in cholesterol biosynthetic

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[1] Abbreviations: EPR, electron paramagnetic resonance; CHO, Chinese hamster ovary; SREBP, sterol regulatory element binding proteins; ^{15}N-PDT, 4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-1-^{15}N-oxyl; LiPc, lithium phthalocyanine; CD, methyl-β-cyclodextrin; CHL−CD, cholesterol−methyl-β-cyclodextrin complex.

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pathways are controlled at the transcriptional level by the transcription factors called SREBPs (*38*). The 25 RA CHO cells, selected for resistance to the water-soluble cholesterol analogue 25-hydroxycholesterol, have significantly higher cellular cholesterol levels (*39*). These cells contain a gain of function mutation in the protein called the SREBP cleavage-activating protein (SCAP), rendering them resistant to downregulation by cholesterol at the transcriptional level (*40*). In a lipoprotein-containing medium, 25 RA CHO cells continue to take in cholesterol via LDL receptors and synthesize cholesterol via biosynthetic pathways, resulting in increased levels of cholesterol (*40*). In contrast, M 19 CHO cells, selected for resistance to amphotericin B, which has a high affinity toward cholesterol in the plasma membrane (*41*), have low cholesterol due to the deletion of the gene encoding the protease that cuts the sterol regulatory element binding proteins (SREBP) at site 2. Therefore, they are unable to release mature SREBP from the endoplasmic reticulum membranes (*42*). Thus, the M 19 cells do not induce the expression levels of a low-density lipoprotein receptor and various cholesterol biosynthetic enzymes such as HMG-CoA reductase and HMG-CoA synthase upon sterol deprivation. As a result, these cells are unable to synthesize cholesterol and unsaturated fatty acids and have an absolute growth requirement for exogenous cholesterol (*41*, *42*).

We also used various biochemical means to alter further the plasma membrane cholesterol concentration and investigated the effect of these modifications on extra- and intracellular oxygen concentrations. The effect of menadione-induced stimulation of oxygen consumption rates on the oxygen gradient also was studied. The oxygen concentration and respiration rates were measured by EPR oximetry. The distribution of cellular cholesterol in various cell types under different conditions was examined with confocal fluorescence microscopy, and the concentration was measured by a microenzymatic fluorescence assay. The results indicate that the cholesterol concentration in the plasma membrane is an important factor affecting the oxygen concentration within CHO cells.

MATERIALS AND METHODS

Materials. Ham's F-12 medium and penicillin−streptomycin (10000 IU/mL and 10000 μg/mL) were purchased from Mediatech Inc. (Herndon, VA), and fetal bovine serum (FBS) was purchased from Gibco Life Technologies (Grand Island, NY). Lithium phthalocyanine (LiPc) was synthesized in our laboratory, and 4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-1-^{15}N-oxyl (^{15}N-PDT) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). The gadolinium complex (magnevist−GdDTPA) was obtained from Berlex Imaging (Wayne, NJ). A slow-fade antifade light kit was obtained from Molecular Probes (Junction City, OR). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Cell Culture. Wild-type Chinese hamster ovary (CHO) cells were obtained originally from ATCC (Manassas, VA). The mutant CHO cell lines were developed and maintained in Dr. Chang's laboratory at Dartmouth Medical School. All of the cell lines were grown as monolayers in Ham's F-12 medium, supplemented with 10% fetal bovine serum (v/v) and 1% penicillin−streptomycin. The cells were cultured at 37 °C in an incubator supplemented with 95% air and 5% CO_2. The ability of cells to exclude 0.4% trypan blue was determined using a hemocytometer under a light microscope and was found to be above 95% in all the experiments, both before and after the measurements by EPR.

Fluorescence Measurement of Cholesterol Distribution. Filipin was used to identify cellular cholesterol as described by Park et al. (*43*). The cells on cover slips were rinsed with ice-cold PBS and were fixed with 1% glutaraldehyde on ice for 15 min. These were rinsed three times with PBS and incubated with filipin for 30 min at room temperature. The cells were washed again and then mounted on a slow-fade antifade light kit (Molecular Probes, Eugene, OR). The fluorescence was observed with confocal fluorescence microscopy (maximum absorption at 358 nm and emission at 461 nm).

Incorporation and Depletion of Plasma Membrane Cholesterol. Free cyclodextrins can selectively deplete cholesterol from isolated or intact cellular membranes, while cholesterol−methyl-β-cyclodextrin complexes (CHL−CD) can function as a cholesterol delivery vehicle to incorporate cholesterol into the cell membrane (*44*−*46*). A commercially available cholesterol−methyl-β-cyclodextrin complex (cholesterol:CD, 53 mg:1 g) was added into the culture media (0.1 mmol/L cholesterol) and incubated with cells for 12 h to incorporate the cholesterol in the plasma membrane. To deplete the cholesterol, the cells were incubated with 5 mmol/L methyl-β-cyclodextrin (CD) for 2 h. Dilipidated serum (CPSR-1) also was used in place of a normal medium for 12 h to remove the cellular cholesterol. Just before the experiments, the cells were washed three times by PBS and were resuspended in Ham's F-12 medium. Fluorescence measurements were carried out to identify the distribution of the cellular cholesterol and also to confirm the change in cholesterol concentration after the chemical treatments.

Lipid Analysis. Free and total cell cholesterol was measured with a microenzymatic fluorescence assay (*47*). The cellular lipids were extracted with chloroform−methanol (2:1 v/v) solution. The assay used for total cholesterol evaluation was composed of potassium phosphate buffer (0.1 M, pH 7.4), cholesterol oxidase (1 unit/mL), horseradish peroxidase (10 units/mL), cholesterol ester hydrolase (0.1 unit/mL), Triton X-100 (0.5%), sodium cholate (20 mM), and *p*-hydroxyphenylacetic acid (4 mg/mL). For analysis of the free cholesterol, the same assay was used but without cholesterol ester hydrolase. The samples were incubated with the assay solution for 30 min at 37 °C, and the fluorescence was measured with a Hitachi fluorescence spectrophotometer Model F-3010 (excitation 325 nm; emission 415 nm). The cellular protein was determined with Lowry's method (*48*).

Measurement of Respiration Rates. Each 100 μL sample of cell suspension (2.5×10^6 cells/mL) was mixed with 10% dextran and 0.2 mM ^{15}N-PDT. The cell suspension was drawn into a glass capillary tube, which was sealed at both ends with Citroseal sealant and placed in the EPR resonator. Oxygen consumption by the cells resulted in a decrease in the oxygen concentration in the sealed tube, which caused a decrease in the line width of the ^{15}N-PDT. Spectra were recorded at 30 s intervals for 10 min, and the change in line widths was transformed to oxygen concentration using the calibration curve. Slopes of the decrease in oxygen concen-

tration versus time yielded the oxygen consumption rates of the cells. A similar procedure was used to measure the respiration rate of cells treated with menadione sodium bisulfite (MSB), CD, CHL−CD, and CPSR-1.

Measurement of Oxygen Concentrations. Samples (200 μL) containing 2.5×10^7 cells/mL, 0.2 mM ^{15}N-PDT, 0.25 mg/mL LiPc, 10% dextran, and 50 mM Gd−DTPA complex were prepared (all the concentrations mentioned are the final concentrations in the cell suspension). The cell suspension was mixed quickly but gently and drawn into a gas permeable Teflon tube with an inside diameter of 0.813 mm and a wall thickness of 0.038 ± 0.014 mm (Zeus Industries, Raritan, NJ). The tube was folded into a W shape and inserted in a quartz tube open at both ends. The addition of the positively charged Gd−DTPA complex broadened the extracellular ^{15}N-PDT signal, and the remaining signal provided a direct measurement of the average intracellular oxygen. At this concentration, the EPR signal arising from the Gd−DTPA complex was too broad to be observed under the present experimental conditions. Because the unpaired electrons in LiPc are located deep within the crystal and hence physically shielded from the media, the Gd−DTPA complex did not affect the line width of LiPc, and the LiPc remained extracellular due to its size. Therefore, LiPc reported exclusively the average extracellular pO_2. The EPR signals of LiPc and ^{15}N-PDT did not overlap. Measurements were carried out at various perfused oxygen concentrations, and the line widths were calculated by spectral fitting, using the EWVoigt program (Scientific Software, Urbana, IL). For calibration, the cellular oxygen consumption was inhibited by 0.5 mM DPI (diphenylpicryl iodide). It is assumed that, in the absence of oxygen consumption, equilibrium exists for oxygen between the extra- and intracellular compartments and could be related to the oxygen tension of the perfused gas. A plot of the oxygen concentration in the medium against the line widths provided the correlation equation, which was used to transform line widths into the oxygen concentrations.

EPR Measurements. All of the experiments were carried out at 37 °C on a Varian E-109 EPR spectrometer equipped with a Varian gas-flow temperature controller. Representative spectroscopic parameters were as follows: microwave frequency, 9.35 GHz; center field, 3320 G for ^{15}N-PDT and 3338 G for LiPc; and modulation frequency, 25 kHz for LiPc and 100 kHz for ^{15}N-PDT. The oxygen concentration of the perfused gas was always verified with an oxygen analyzer (Delta F Corp., Woburn, MA) before the experiments.

Statistic Analysis. The data are expressed as mean ± SD and were analyzed by ANOVA. Statistical significance was accepted at $p < 0.05$.

RESULTS

Cholesterol Distribution in Wild-Type CHO and Mutant Cells. The phase contrast and filipin staining images are shown in panels A and B of Figure 1, respectively. In all of the cell types, most of the fluorescence originated from the plasma membranes, which is consistent with the earlier reports (*49*). The fluorescent intensity varied according to the cholesterol content in these cell lines and was 25 RA > wild-type > M 19 CHO cells. Treatment with CPSR-1 led to a significant decrease of fluorescence in wild-type and 25 RA CHO cells. A similar effect was observed in cells treated with CD. No significant change was observed in M 19 CHO cells treated with CD or CPSR-1. We also found that the fluorescence significantly increased in all of the cells exposed to a CHL−CD complex for 12 h.

Cholesterol Concentration in Wild-Type CHO and Mutant Cells. A microenzymatic fluorescence assay of free and total cholesterol was used to measure the cholesterol concentration in the cells. As shown in Table 1, the free and total cholesterol levels of 25 RA CHO cells were significantly higher than those of the wild-type and M 19 CHO cells. Incubation of the cells with a CHL−CD complex resulted in a significant increase in both the free and total cholesterol concentration in all of the cell lines. Treatments with either CD or CPSR-1 significantly decreased the total and free cholesterol concentration in wild-type and 25 RA CHO cells but not in M 19 CHO cells.

Oxygen Consumption Rates in Wild-Type, 25 RA, and M 19 CHO Cells. Oxygen consumption rates of 25 RA and M 19 CHO cells were not significantly different from that of wild-type CHO cells (Table 2). However, the oxygen consumption rates of M 19 CHO cells were significantly lower (∼13%) than those of 25 RA CHO cells. Oxygen consumption rates of the wild-type CHO cells were significantly reduced (∼27%) when treated with the CHL−CD complex, but no significant change was observed with treatment with CD or CPSR-1. Exposure of 25 RA CHO cells to CD, CPSR-1, or CHL−CD reduced the oxygen consumption rates significantly. CD or CHL−CD treatments decreased oxygen consumption rates of M 19 CHO cells significantly, while CPSR-1 showed no significant effects. Menadione stimulated the oxygen consumption rates of all the cell lines in a dose-dependent way (Figure 2). The maximum stimulation of the oxygen consumption rate by menadione was observed at a concentration of 50−200 μmol/L. In viable cells MSB is metabolized and then reacts with oxygen, generating superoxide, consuming oxygen, and potentially causing oxidative stress (*50*−*52*). At high concentrations of menadione, cell viability decreased (as reflected by the ability to exclude the trypan blue test), which resulted in a decrease in oxygen consumption rate of the cells.

Oxygen Gradients in Wild-Type, 25 RA, and M 19 CHO Cells. In all three cell lines, the extracellular concentration of oxygen measured with three different concentrations of perfused gas did not change significantly in controls and after various treatments, but the intracellular concentration of oxygen varied after the various treatments (Table 3). The resulting oxygen gradients (difference between extra- and intracellular oxygen concentration) observed in wild-type, 25 RA, and M 19 CHO cells under various conditions are shown in Figure 3. The results indicate that the type of cells and the concentration of oxygen perfusing the sample affected the magnitude of the oxygen gradient. It also was significantly affected by the various treatments used to alter the cholesterol concentration in the plasma membrane and stimulation of respiration rates. The gradient of oxygen concentration observed after various treatments is compared with that of the controls in Table 4. The oxygen gradients observed in the 25 RA CHO cells were significantly different, with the order as 25 RA CHO > wild-type CHO > M 19 CHO cells at all perfused oxygen concentrations. Treatment of 25 RA and wild-type CHO cells with CPSR-1 or CD

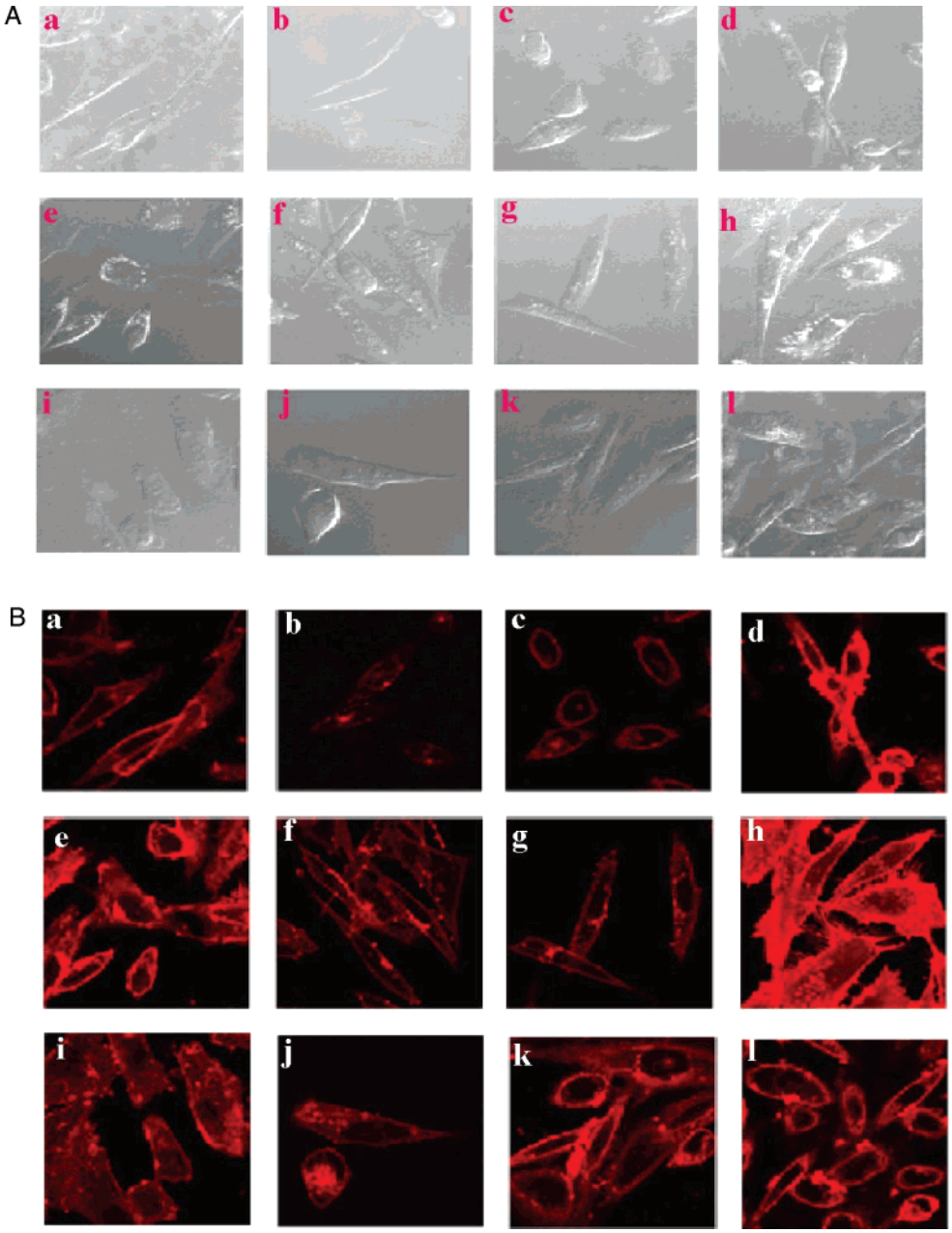

FIGURE 1: Fluorescence measurements of cholesterol distribution in wild-type, 25 RA, and M 19 CHO cells: (A) phase contrast images and (B) fluorescence images. Cells were also treated with 5 mmol/L methyl-β-cyclodextrin (CD), 10% CPSR-1, and the cholesterol−methyl-β-cyclodextrin complex (CHL−CD), respectively. The symbols in both panel A and panel B refer to (a) wild-type CHO cells (WT), (b) WT + CD, (c) WT + CPSR-1, (d) WT + CHL−CD, (e) 25 RA cells (25 RA), (f) 25 RA + CD, (g) 25 RA + CPSR-1, (h) 25 RA + CHL−CD, (i) M 19 cells (M 19), (j) M 19 + CD, (k) M 19 + CPSR-1, and (l) M 19 + CHL−CD.

Table 1: Free and Total Cholesterol Concentration (μg/mg of Protein) in Wild-Type, 25 RA, and M 19 CHO Cells Measured by Microenzymatic Fluorescence Assay[a]

	wild-type CHO		25 RA CHO		M 19 CHO	
	free	total	free	total	free	total
control	20.9 ± 3.3	25.9 ± 4.5	59.1 ± 4.3	134.7 ± 11.9	9.5 ± 0.9	13 ± 1.9
CPSR-1	10.9 ± 4.9	14.3 ± 2.8	17.9 ± 6.1	52.1 ± 10.1	7.5 ± 2.3	9.7 ± 1.3
CD	11.9 ± 4.7	17.7 ± 1.9	15 ± 0.9	51.7 ± 7.7	9.3 ± 1.7	11.3 ± 1.7
CHL−CD	64.6 ± 20.8	88.3 ± 8.7	225.9 ± 19.9	301.7 ± 25.7	43.7 ± 8.1	47.7 ± 2.5

[a] Cells were treated with CPSR-1 (10%), methyl-β-cyclodextrin (CD, 5 mmol/L), and the cholesterol−methyl-β-cyclodextrin complex (CHL−CD, 0.1 mmol/L cholesterol). Mean ± SD, $n = 7-9$.

reduced the oxygen gradient significantly at 210 and 100 μM perfused oxygen. In M 19 CHO cells, CD reduced the oxygen gradients observed at 210 and 100 μM, while a significant decrease in CPSR-1-treated cells was observed at 50 and 100 μM perfused gas. The oxygen gradients observed at 210 μM perfused gas increased significantly when all of the cell lines were treated with a CHL−CD complex. Menadione significantly increased the oxygen

Table 2: Oxygen Consumption Rates (nmol per 10^6 cells per min) in Wild-Type, 25 RA, and M 19 CHO Cells[a]

	wild-type CHO	25 RA CHO	M 19 CHO
control	1.39 ± 0.05	1.45 ± 0.03	1.26 ± 0.05
CPSR-1	1.29 ± 0.03	0.93 ± 0.13	1.29 ± 0.07
CD	1.27 ± 0.03	0.97 ± 0.05	1.08 ± 0.05
CHL−CD	1.02 ± 0.02	0.99 ± 0.02	0.75 ± 0.01

[a] Cells were treated with methyl-β-cyclodextrin (CD, 5 mmol/L), CPSR-1 (10%), and the cholesterol−methyl-β-cyclodextrin complex (CHL−CD, 0.1 mmol/L cholesterol). Mean ± SD, $n = 7-9$.

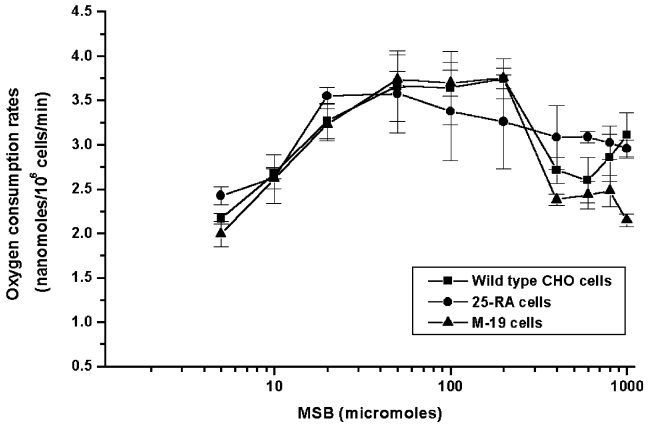

FIGURE 2: Effects of menadione sodium bisulfite (MSB) on oxygen consumption rates (nanomoles per 10^6 cells per minute) of wild-type, 25 RA, and M 19 CHO cells. Mean ± SD, $n = 16$.

gradient in wild-type and 25 RA CHO cells at 210 μM perfused oxygen, but no significant change was observed in M 19 CHO cells.

DISCUSSION

The results from confocal fluorescence microscopy and the microenzymatic assay are consistent with the different plasma membrane cholesterol concentrations in wild-type, 25 RA, and M 19 CHO cells and the effect of the various treatments. M 19 CHO cells maintain a very low concentration of cellular cholesterol (*41*, *42*), and therefore no significant change was observed when these cells were treated with cholesterol depleting agents, CD or CPSR-1. However, exposure to a CHL−CD complex increased the fluorescence and cholesterol concentration in all of the cell lines, indicating increased incorporation of cholesterol in the plasma membrane. Treatments with cholesterol-modifying agents caused some statistically significant, but only modest changes in the oxygen consumption rates. The different levels of oxygen gradient observed in wild-type, 25 RA, and M 19 CHO cells correlate well with the different plasma membrane cholesterol concentration in these cells: 25 RA > wild-type > M 19 CHO cells. This is further supported by (a) the decrease in the oxygen gradient when the plasma membrane cholesterol was depleted by CD or CPSR-1 and (b) the increase in the oxygen gradient when the plasma membrane cholesterol concentration was increased by the CHL−CD complex. The increase or decrease in oxygen gradients that was observed cannot be accounted for by the small variations observed in the oxygen consumption rates after the treatments with CD, CPSR-1, or CHL−CD.

No significant change was observed in the extracellular concentration of oxygen in control and after various treatments (Table 3). This indicates that the oxygen gradients obtained are not due to different oxygen concentrations in the extracellular medium. Although our results on the changes in oxygen gradients after various treatments were more consistent at 210 μM perfused oxygen, some similar significant changes also were seen at lower concentrations of perfused oxygen.

It is reasonable to expect that there would be evolutionary pressure for cells to control the level of oxygen, because it is required for normal processes and potentially is toxic. Therefore, cells would benefit from mechanisms that prevent oxygen from reaching undesirable levels while maintaining sufficient levels to facilitate essential oxygen requiring reactions. The mechanisms for controlling these gradients across the cell membrane will necessarily be reflected in the structure of cells, and alterations in these mechanisms are likely to result in pathophysiology. These results indicate

Table 3: Extra- and Intracellular Concentration of Oxygen (μM) in Wild-Type, 25 RA, and M 19 CHO Cells: Control and after Treatments with CPSR-1, CD, CHL−CD, and Menadione[a]

	wild type		25 RA		M 19	
	$[O_2]_{extr}$	$[O_2]_{intr}$	$[O_2]_{extr}$	$[O_2]_{intr}$	$[O_2]_{extr}$	$[O_2]_{intr}$
			Perfused Oxygen: 210 μM			
control	228.2 ± 12.5	181.3 ± 9.3	223.3 ± 10.3	159.4 ± 7.3	225.1 ± 12.9	205.1 ± 6.5
CPSR-1	225.1 ± 2.6	212.2 ± 8.7	213.7 ± 1.7	209.1 ± 8.1	221.9 ± 5.3	195.5 ± 6.7
CD	217.7 ± 2.8	199.5 ± 13.2	220.5 ± 3.7	200.2 ± 8.2	222.1 ± 4.1	215.5 ± 14.7
CHL−CD	222.7 ± 4.4	169.1 ± 16.0	223.9 ± 5.7	142.5 ± 6.9	220.7 ± 2.8	147.1 ± 10.9
MSB	222 ± 12.3	158.9 ± 7.9	218.1 ± 4.1	117.5 ± 7.7	215.8 ± 6.1	196.5 ± 15.6
			Perfused Oxygen: 100 μM			
control	107.1 ± 7.9	79.8 ± 3.8	101.1 ± 4.5	68 ± 4.5	105.5 ± 4.7	91.1 ± 0.3
CPSR-1	116.1 ± 4.6	95.5 ± 9.5	105.3 ± 4.7	100 ± 1.1	112.1 ± 2.1	102.7 ± 2.9
CD	104 ± 2.3	84.7 ± 5.7	109.1 ± 1.5	86.1 ± 3.3	111.7 ± 0.3	102.8 ± 9.5
CHL−CD	104.3 ± 1.1	88.5 ± 7.1	105.7 ± 1.7	64.7 ± 4.5	104.9 ± 1.2	99.7 ± 7.9
MSB	95.5 ± 4.0	84.9 ± 16.3	110.7 ± 1.8	57.1 ± 5.5	90.7 ± 4.71	89.7 ± 19.9
			Perfused Oxygen: 50 μM			
control	54 ± 4.0	47.9 ± 1.7	52 ± 2.9	43.1 ± 3.9	51.5 ± 2.1	49.9 ± 7.2
CPSR-1	52.7 ± 1.4	45.5 ± 8.1	55 ± 4.5	44.7 ± 0.5	62.9 ± 3.8	61.6 ± 1.7
CD	51.2 ± 0.8	43 ± 7.3	52.6 ± 1.3	41.2 ± 4.3	52.1 ± 0.4	34.5 ± 16.9
CHL−CD	49.3 ± 0.5	37.7 ± 12.9	49.9 ± 1.1	40.5 ± 2.9	50.2 ± 0.9	35.7 ± 3.5
MSB	43.1 ± 12.5	38.4 ± 1.9	49.6 ± 2.1	39.1 ± 2.7	46.6 ± 9.3	30.52 ± 3.3

[a] Mean ± SD, $n = 7-9$.

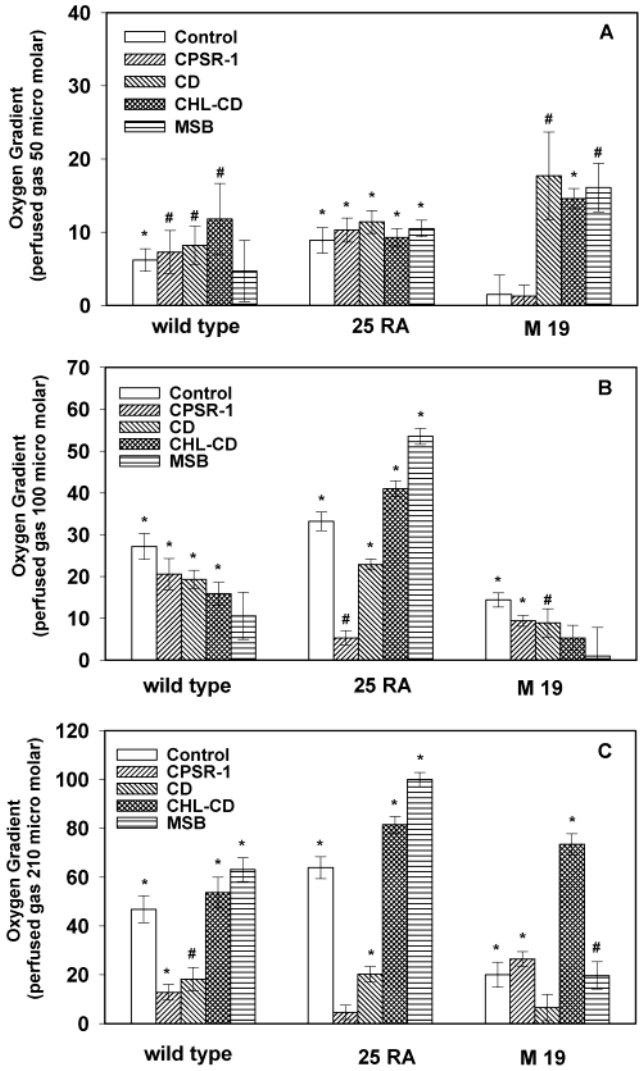

FIGURE 3: Oxygen gradient (μM) between extra- and intracellular compartments of cells at different perfused oxygen concentrations: (A) 50 μM perfused gas, (B) 100 μM perfused gas, and (C) 210 μM perfused gas. Please note the difference in the scale of the oxygen gradient plotted for each perfused gas concentration. For the occurrence of an oxygen gradient (*) $p \leq 0.001$ and (#) $p < 0.05$.

Table 4: Difference in Gradients of Oxygen (μM) between Control and Various Treatments at Each Perfused Oxygen Concentration[a]

CHO cell line	sample	perfused [O_2] 50 μM	100 μM	210 μM
wild type	CPSR-1	1.1 (0.348)	−6.68 (<0.001)	−34.0 (<0.001)
	CD	2.01 (0.061)	−7.93 (<0.001)	−28.6 (<0.001)
	CHL−CD	5.56 (0.003)	−11.39 (<0.001)	6.86 (0.025)
	MSB	−1.45 (0.335)	−16.69 (<0.001)	16.26 (<0.001)
25 RA	CPSR-1	1.37 (0.104)	−27.9 (<0.001)	−59.24 (<0.001)
	CD	2.53 (0.002)	−10.27 (<0.001)	−43.62 (<0.001)
	CHL−CD	0.50 (0.512)	7.85 (<0.001)	17.63 (<0.001)
	MSB	1.58 (0.0288)	20.34 (<0.001)	36.6 (<0.001)
M 19	CPSR-1	−0.15 (<0.889)	−5.03 (<0.001)	6.38 (0.002)
	CD	16.17 (<0.001)	−5.56 (<0.001)	−13.49 (<0.001)
	CHL−CD	13.04 (<0.001)	−9.18 (<0.001)	53.48 (<0.001)
	MSB	14.58 (<0.001)	−13.41 (<0.001)	−0.64 (0.805)

[a] Numbers in parentheses indicate the p value for the occurrence of an oxygen gradient as compared to controls.

that the gradient is affected by a plausible mechanism, the concentration of cholesterol in the plasma membrane of the cell. Changes in the concentration of cholesterol in the membrane would stress the ability of the cell to maintain an appropriate gradient.

The oxygen gradients of the magnitudes that were observed cannot be accounted for on the basis of simple passive diffusion of oxygen into the interior of oxygen consuming cells, and therefore these results suggest that the cholesterol in the plasma membrane causes a significant "barrier" to the diffusion of oxygen into the intracellular compartments. A significant increase in the oxygen gradient of 25 RA and wild-type CHO cells on menadione stimulation but no change in M 19 CHO cells provides further evidence that the oxygen gradients observed in these cell lines are not a simple reflection of the cellular oxygen consumption rates but are most likely due to different plasma membrane cholesterol concentrations.

In conclusion, these results indicate that the cholesterol concentration in the plasma membrane is an important factor for restricting the oxygen diffusion across the cell membrane and hence is involved in the maintenance of an oxygen gradient in cells.

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