Appl. Magn. Reson. 22, 357–368 (2002)

Applied Magnetic Resonance

Development of Isoindoline Nitroxides for EPR Oximetry in Viable Systems

J. Shen[1], S. Bottle[2], N. Khan[1], O. Grinberg[1], D. Reid[2], A. Micallef[2], and H. Swartz[1]

[1] EPR Center, Department of Diagnostic Radiology, Dartmouth Medical School, Hanover, New Hampshire, USA
[2] Center for Instrumental and Developmental Chemistry, Queensland University of Technology, Brisbane, Queensland, Australia

Received July 7, 2001; revised January 11, 2002

Abstract. Nitroxides are widely used as biophysical probes to study molecular motion, intracellular oxygen, pH, transmembrane potential, and cellular redox metabolism, etc. They may be rapidly metabolized to hydroxylamines by cells, which limits their use in viable systems. In this study, we have characterized relevant properties in cells of several isoindoline nitroxides that have been prepared to have different physicochemical properties: 1,1,3,3-tetramethylisoindolin-2-yloxyl (TMIO) and its analogs 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (CTMIO), 5-(N,N,N-trimethylammonio)-1,1,3,3-tetramethyl isoindolin-2-yloxyl iodide (QATMIO) and 2-hydroxy-1,1,3,3-tetramethylisoindoline hydrochloride (TMIOH.HCl). The oxygen sensitivity and metabolic kinetics of these were compared in CHO cells under different oxygen tensions with 1-oxyl-2,2,6,6-tetramethyl-4-piperidione (Tempone) and 3-carboxyl-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (PCA). Cytotoxicity was evaluated by the measurement of oxygen consumption rates, trypan blue exclusion, and clone formation. TMIO and its analogues have a higher relative oxygen sensitivity than Tempone and PCA with the oxygen sensitivity in electron paramagnetic resonance (EPR) spectrometry in the order of: TMIO = TMIOH = CTMIO > QATMIO = Tempone < PCA. The rates of metabolism of these nitroxides are moderate and depend on oxygen concentration, ring type, ring substituent, and membrane permeation. These nitroxides have low cytotoxicity. The results indicate that TMIO and its analogues are potentially useful for EPR studies of viable systems, especially for oximetry.

1 Introduction

Nitroxides are frequently used as paramagnetic labels in electron paramagnetic resonance (EPR, or completely equivalently, electron spin resonance, ESR) techniques to study biophysical parameters, such as the concentration of oxygen [1–6], pH [7], membrane fluidity [8, 9], cellular metabolism [10], and structural properties of membrane transport proteins [11]. The nitroxides also are potential contrast agents for in vivo nuclear magnetic resonance (NMR) imaging studies [12, 13] and pharmaceutical agents for monitoring drug release [14]. Furthermore, nitroxides and their

hydroxylamines have been suggested as potential therapeutic or diagnostic drugs on the basis of their superoxide dismutase mimicking function [15–18] and interactions with free radicals such as superoxide radicals and peroxynitrite [19, 20]. When used in model systems, nitroxides are quite stable. In viable systems, however, they are metabolized to the corresponding EPR-silent hydroxylamines. Our goal is to develop isoindoline nitroxides to achieve better stability and oxygen sensitivity in viable systems, especially for use in EPR oximetry.

The use of nitroxides for EPR oximetry is a rapidly growing field. Oxygen broadens the EPR spectral line width of nitroxides via Heisenberg spin exchange [21, 22] and this property is used to determine oxygen concentration. In previous studies, nitroxides on the basis of piperidine and pyrrolidine rings have been used to measure oxygen concentrations in biological systems [1–3, 5, 14, 23, 24]. We have been using EPR oximetric techniques to measure intra- and extracellular oxygen concentration in cell suspensions, in order to determine whether significant gradients can occur [1–3, 23, 24].

With EPR and other techniques, it has been demonstrated that cell metabolism reduces nitroxides to nonparamagnetic hydroxylamines [25–30]. Cellular systems can also oxidize hydroxylamines to nitroxides [25, 31]. The bioreduction of nitroxides depends on their physicochemical properties, especially the nature of the ring in which the nitroxide moiety is located, the lipophilicity or hydrophilicity of the nitroxide, and the charge on the molecule [25]. In addition, cells under different physiological and pathophysiological conditions, such as different oxygen concentrations [29], cellular redox metabolic state [10], and in the presence of oxidizing agents [32], may have different rates of nitroxide metabolism. It is therefore desirable to develop nitroxides on the basis of other rings that may have enhanced properties for particular uses in biological systems, including EPR oximetry.

In this study, we evaluated several nitroxides on the basis of the isoindoline ring with different physicochemical properties: a lipid-soluble nitroxide 1,1,3,3-tetramethylisoindolin-2-yloxyl (TMIO), its neutral carboxy analogue 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (CTMIO), its charged trimethyl ammonium analogue 5-(N,N,N-trimethylammonio)-1,1,3,3-tetramethylisoindolin-2-yloxyl iodide (QATMIO), and its hydroxyl amine 2-hydroxy-1,1,3,3-tetramethylisoindoline hydrochloride (TMIOH.HCl). In order to identify their potential for use in EPR oximetry, the relative oxygen sensitivities and bioreduction kinetics were compared with two commercially available and widely employed nitroxides, 1-oxyl-2,2,6,6-tetramethyl-4-piperidione (Tempone) and 3-carboxyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (PCA), in CHO cells. In addition, the cytotoxicity of these nitroxides on CHO cells was measured by trypan blue exclusion, clonogenicity, and effects on oxygen consumption.

2 Materials and Methods

Reagents. TMIO, CTMIO, QATMIO and TMIOH.HCl were synthesized at the Queensland University of Technology, Australia, by published synthetic proce-

dures [33–35]. 4-oxo-2,2,6,6-tetramethylpiperidine-d16-1-^{15}N-oxyl (^{15}N-PDT) was purchased from MSD Isotopes (St. Louis, MO). PCA and Tempone were obtained from Molecular Probes (Junction City, OR). McCoy's 5A, heat-inactivated fetal bovine serum, dextran, HEPES, penicillin, streptomycin, and trypsin were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. Chinese hamster ovary (CHO) cells were seeded in McCoy's 5A medium supplemented with 10% fetal bovine serum, 20 mM HEPES, and 1% penicillin/streptomycin, and cultured in a humidified incubator at 37°C with 95% air and 5% CO_2. The cells were maintained as monolayers and subcultured three times before each experiment.

Clonogenicity Assay. The cells were collected by trypsinization (0.25% trypsin), centrifuged (200 g, 5 min), and then seeded into 12-well, round-bottom sterile plates at a concentration of 200 cells/well. Different concentrations (0.1, 0.5, 1.0 mM, final concentration) of the nitroxides were added into the culture media. After incubation for 36 h, the cells were washed 3 times with HEPES and cultured with fresh media. After being cultured for 7 days, the cells were fixed and observed under a microscope to count visible colonies. The effects of the nitroxides and TMIOH on colony formation were calculated by comparing with untreated controls.

Trypan Blue Exclusion Test. The effects on cell integrity were measured by the trypan blue exclusion test. Different concentrations (0.1, 0.5, 1.0 mM, final concentration) of the nitroxides and TMIOH were added into culture media and incubated at 37°C in a humidified incubator with 95% air and 5% CO_2 for 24 h. The ability to exclude 0.4% trypan blue was determined with a hemocytometer under a light microscope.

Oxygen Consumption Measurements. The effects on cell function were assessed by measuring the rate of consumption of oxygen. Each 100 ml sample of cells ($2.5 \cdot 10^6$ cells/ml) was mixed with 10% dextran (to retard settling of the cells) and 0.5 mM ^{15}N-PDT. Different concentrations (0.1, 0.5, 1.0 mM, final concentration) of the nitroxides were added into the system. The resulting solution was drawn into a 1 mm (inner diameter) quartz capillary tube that was then sealed at both ends. The EPR spectra were recorded at 30 s intervals, and the rates of oxygen consumption by CHO cells were calculated from the slope of the change in line width of ^{15}N-PDT with time. (During the time required for the assay, the concentration of PDT did not change significantly and therefore the changes in linewidth could be attributed entirely to changes in $[O_2]$.)

Measurement of Reduction Rates of Nitroxides. Nitroxides (0.5 mM, final concentration) were added into McCoy's 5A media in the presence of $2.5 \cdot 10^7$ cells/ml and 10% dextran. Each sample 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 then inserted into a quartz EPR tube open at both ends. The tube was placed in the EPR cavity, and gas of the desired composition was flowed through the resonator. The temperature was maintained at 37°C. The time interval between the addition of the nitroxide and the beginning of data

collection was 2 min. To measure the metabolic rates of the nitroxides in response to the different concentrations of oxygen, the perfused gas was changed from 21% oxygen to 10% and then to 0% oxygen. The EPR spectra were recorded at 1 min intervals and data were collected for 25 min with each perfused gas. The kinetics of the reduction process was derived from the time-dependent changes in the signal intensity of the nitroxides. It has been shown previously that low concentrations of ferricyanide can oxidize hydroxylamines to nitroxides [24]. Ferricyanide (0.5 mM) was added into cell suspensions ($2.5 \cdot 10^7$ cells/ml) containing 0.5 mM TMIOH.HCl. After incubation for 1 min, the EPR spectra were recorded at 21, 10, and 0% perfused oxygen concentrations for 25 min each. In the corresponding control group, the ferricyanide was omitted. The reduction rates of the TMIO produced by the oxidation of TMIOH were calculated as described above.

Measurement of Oxidation of Hydroxylamines to Nitroxides. We studied the oxidation of TMIOH and reduction of the resulting nitroxide at three different cell concentrations. Freshly prepared TMIOH (0.05 mM) was added to a cell suspension with 10% dextran and the increase in signal intensity of the oxidation product, TMIO, was monitored at 21% perfused O_2. The perfused gas was changed to 0% O_2 after 90 min to determine the reduction rates of the resulting nitroxide in the absence of oxygen. In order to analyze the kinetic curves of oxidation of TMIOH and simultaneous reduction of TMIO in cells, we applied the following rate equations:

$$dR/dt = k_1[\mathrm{OH}] - k_2[R] \text{ for oxidation and reduction in 21\% } O_2, \quad (1)$$

$$[\mathrm{OH}] + R = [\mathrm{OH}]_0, \quad (1a)$$

$$dR/dr = -k_3[R] \text{ for reduction in 0\% } O_2, \quad (2)$$

where the first-order rate constants are: k_1 (the rate constant for oxidation), k_2 (the rate constant for reduction at 21% oxygen), and k_3 (the rate constant for reduction at 0% oxygen); $[\mathrm{OH}]_0$ is the concentration of TMIOH at time $t = 0$, [OH] is the concentration of TMIOH at time t, R is the concentration of TMIO in moles/l, derived by EPR.

The rate Eqs. (1) and (2) were solved to obtain the concentration of nitroxides during oxidation and reduction:

$$R = A + k_1[\mathrm{OH}]_0 \frac{1 - \exp(-k_1 - k_2 t)}{k_1 + k_2} \text{ for oxidation and reduction in 21\% } O_2, \quad (3)$$

$$R = B + R_0 \exp(-k_3 t) \text{ for reduction in 0\% } O_2, \quad (4)$$

where A and B are adjustable parameters that reflect nitroxides and hydroxylamines that are not in the pool of reacting molecules (these may reside in the walls of the tubing or other inaccessible sites that are not in equilibrium with

the rest of the species), R_0 is the concentration of nitroxides when the perfused gas was changed from 21 to 0% O_2.

In order to determine the rate constants, the fitting of the experimental kinetic curves was performed with rate Eqs. (3) and (4). The units of these constants are: $[OH]_0 k_1$, moles/l/s; $[k_2]$ and $[k_3]$, 1/s. The relatively low values of A and B, $A \cdot k_2/k_1[OH] = 10.32 \pm 1.55$, $B/R_0 = 15.04 \pm 6.8$ suggest that only a small proportion of the molecules are not in equilibrium with the rest.

Cell integrity was checked by the trypan blue exclusion test after each experiment and was found to be above 95% in all the experiments.

Effects of Freeze-Thawing. To test the hypothesis that the enhanced stability of QATMIO is due to its low membrane permeation, a series of cell suspensions was prepared incorporating three freeze-thaw cycles to lyse the cells. This was achieved by freezing in liquid nitrogen followed by thawing in a water bath at 37°C. Nitroxides (0.5 mM, each) were then added into the freeze-thawed cell suspensions and rates of reduction were measured relative to normal (unlysed) cell suspensions.

Measurement of Sensitivity to Oxygen. Nitroxides (0.5 mM) were added into cell suspensions containing $2.5 \cdot 10^7$ cells/ml and EPR spectra were recorded in different concentrations of perfused gas. The relative sensitivity to oxygen was determined in terms of the change in line width with respect to that in 0% oxygen.

EPR Measurements. The EPR spectra were recorded on a Varian E-109 EPR spectrometer, equipped with a Varian gas-flow temperature controller. Representative spectroscopic parameters were: field center, 3320 Gauss; frequency, 9.34 GHz; modulation amplitude, 0.1 Gauss; and nonsaturating microwave power. To derive the line width, the lower-field component ($N = 1$) of the EPR signal was fitted with the EWVoigt program (Scientific Software, IL), which utilizes a convolution of Lorentzian and Gaussian functions (Voigt function) to describe EPR line shape. The line width of this function was used to describe the effect of oxygen. To fit the line shape of EPR signals without superhyperfine structure, several parameters such as Lorentzian line width, signal intensity, center field, and signal phase were adjusted keeping the Gaussian function constant as approximately 10% of the total line width. To derive the line width of EPR signals with well resolved superhyperfine structure, the signal was fitted with the superhyperfine splitting of 12 protons as an additional adjustable parameter. In our experimental conditions, the four methyl groups showed equivalent splittings. This was determined by a simulation of the spectra, which fitted well when the four methyl groups were assumed equivalent. In our hands, no hyperfine splitting arising from the other ring protons was observed. To fit the spectra of these radicals with unresolved superhyperfine structures at 21% oxygen, the superhyperfine splitting derived at 0% perfused oxygen was used as a nonadjustable parameter to derive the line width. For EPR lines with superhyperfine splittings, the line width of each superhyperfine line was assumed to be the same and the fitting gave the mean line width of the superhyperfine splittings of the $N = 1$ hyperfine component.

Statistical Analysis. All data are expressed as mean with ± standard error (S.E.) and were analyzed by ANOVA. Statistical significance was accepted at $P < 0.05$.

3 Results

3.1 Effects of Oxygen on EPR Spectra

The structures of the nitroxides along with oxygen-induced line width broadening of Tempone and TMIO are shown in Fig. 1. The isoindolines such as TMIO had resolvable superhyperfine splittings at low oxygen concentrations, which broadened at higher oxygen levels. TMIOH.HCl had no EPR signal initially; however, a signal with the characteristic splittings of TMIO developed slowly with time, indicating its oxidation to TMIO. None of the nitroxides showed any reduction in medium alone. All of the nitroxides had stable line widths during the measurements in each perfused gas.

In order to determine the suitability of these isoindoline nitroxides for use in EPR oximetry, the changes in their line widths were compared with those of Tempone and PCA in cell systems under different oxygen concentrations in the

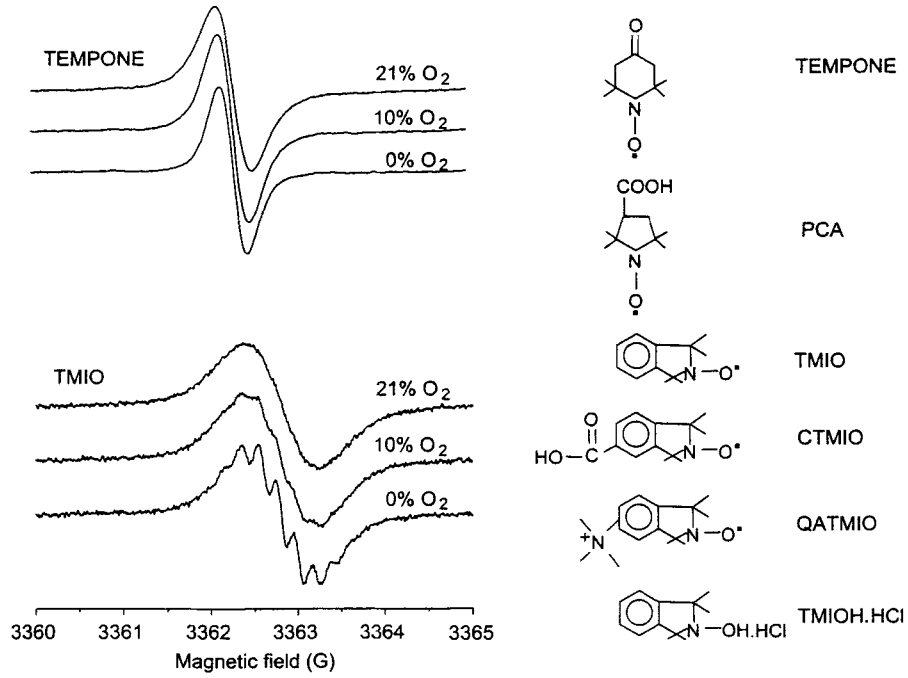

Fig. 1. Chemical structures of TMIO, CTMIO, QATMIO, TMIOH.HCl, Tempone and PCA and typical EPR spectra of TMIO and Tempone in media exposed to 21, 10, and 0% perfused oxygen.

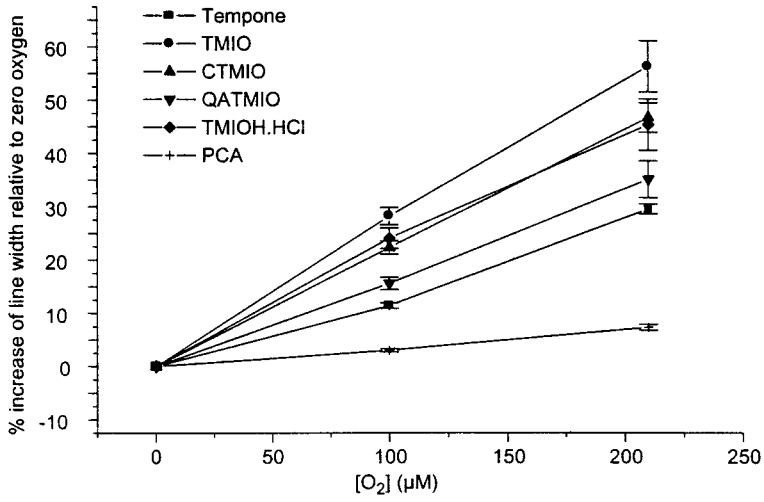

Fig. 2. Increases in line width (% increase of line width compared to line width in the absence of oxygen) in the presence of different perfused oxygen concentrations. % change = $(LW_{O2} - LW_{N2})/LW_{N2} \cdot 100\%$. Data are expressed as mean ± S.E., $n = 3$–5.

perfused gas (Fig. 2). As expected, all the nitroxides showed an oxygen-concentration-dependent line width broadening, summarized in Table 1. While the amount of absolute broadening of line width due to oxygen is similar for all nitroxides, the ability to use the broadening for oximetry depends on the relative changes in line width, because it is easier to measure accurately increments of the same magnitude in a narrower line. The relative sensitivity of the nitroxides to oxygen is shown in terms of the relative change in line width from that at 0% oxygen and was found to be in the order: TMIO = TMIOH = CTMIO > QATMIO = Tempone < PCA.

Since TMIOH.HCl upon oxidation by a mild oxidant such as ferricyanide should be identical to TMIO, some of material was oxidized and the line width was not significantly different from that of the parent TMIO.

Table 1. Line width of the nitroxides at different concentrations of perfused oxygen. Values are means ± S.E.

Nitroxides	Line width (G) at concentration of perfused O_2 of:		
	0%	10%	21%
TMIO	0.19±0.01	0.24±0.01	0.29±0.01
CTMIO	0.21±0.01	0.25±0.01	0.30±0.01
QATMIO	0.24±0.02	0.28±0.02	0.33±0.01
Tempone	0.33±0.01	0.37±0.01	0.43±0.01
PCA	0.83±0.01	0.86±0.01	0.90±0.01

3.2 Metabolism of Nitroxides in Intact and Freeze-Thawed CHO Cells under Different Oxygen Concentrations

The reduction rates of the nitroxides in intact and freeze-thawed cells under 0, 10, and 21% perfused oxygen are shown in Fig. 3. Compared with Tempone, TMIO had similar reduction rates. CTMIO, however, had lower reduction rates in the intact cell system under 21% oxygen. The reduction rates of the nitroxides in 10% oxygen were not significantly different from those in 21%, suggesting that oxygen concentration in this range has little influence on the metabolism of nitroxides.

When the system was exposed to zero oxygen, the reduction rates of the nitroxides increased significantly. This observation is consistent with previous studies [29, 36, 37]. The reduction rates of TMIO were higher than Tempone, but CTMIO had lower rates.

The charged isoindoline nitroxide QATMIO was much more resistant than the other isoindolines to reduction in this cellular system. This is consistent with the expectation that most of the reduction occurs within cells and the charged species does not readily cross intact cell membranes [28, 36, 37].

The reduction rates of TMIO generated from oxidation of TMIOH by ferricyanide (0.5 mM) were similar to that of parent TMIO (TMIO: 21% O_2, 1.84±0.15; 10% O_2, 2.33±0.21; 0% O_2, 6.28±0.36; TMIO oxidized from TMIOH: 21% O_2, 1.47±0.21; 10% O_2, 2.40±0.18; 0% O_2, 7.12±0.88), indicating that the cells produced the same product from TMIOH, as expected.

We investigated the reduction rates in freeze-thawed CHO cells to determine if the resistance to reduction of QATMIO was due to its failure to cross cell

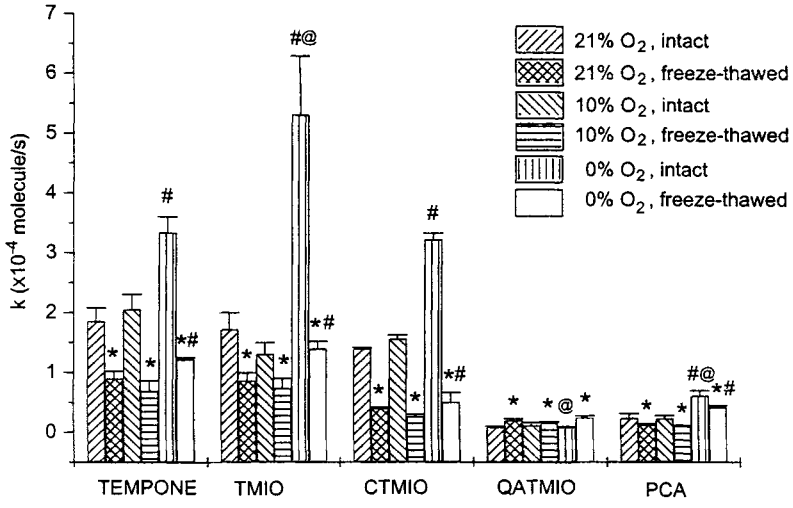

Fig. 3. Apparent reduction rates of nitroxides in intact and freeze-thawed CHO cells under 0, 10, 21% O_2 (mean ± S.E., $n = 3$–5). * $P < 0.05$, vs. normal cells; # $P < 0.05$, vs. rate at 21% O_2; @ $P < 0.05$, vs. Tempone in normal cells under 0% O_2.

membranes. The rates of reduction in freeze-thawed cells were lower than those of intact cells for all of the nitroxides (except for QATMIO), indicating that freeze-thawing decreased the ability of these cells to reduce nitroxides. In contrast, the reduction rate of QATMIO did not decrease in freeze-thawed cells (in fact, the rate was significantly increased in the freeze-thawed cells). These results are consistent with the expectation that low membrane permeation of QATMIO accounts for its resistance to reduction by the intact cells.

3.3 Oxidation of Hydroxylamine to Nitroxide

The use of nitroxides in biological systems has led to a need to understand thoroughly the reduction and oxidation of nitroxides in living cells. The predominant reaction is known to be a reversible reduction to hydroxylamines [25–32,

Table 2. Effects of the nitroxides on trypan blue exclusion rates, clonogenicity rates and oxygen consumption rates in CHO cells.

Treatment of cells	Trypan blue exclusion (%)	Clonogenicity (%)	Oxygen consumption (nmoles/10^6 cells/min)
Control	99.3±0.2	57.8±3.1	2.6±0.2
Tempone			
0.1 mM	99.3±0.2	59.2±2.5	2.5±0.3
0.5 mM	98.8±0.2	56.2±1.3	2.9±0.2
1.0 mM	98.7±0.2	51.3±2.5	2.2±0.3
PCA			
0.1 mM	99.4±0.1	57.5±2.5	2.5±0.2
0.5 mM	99.3±0.1	54.1±2.4	2.7±0.4
1.0 mM	97.3±1.9	55.5±1.9	2.2±0.1
TMIO			
0.1 mM	99.1±0.3	52.8±2.7	2.7±0.1
0.5 mM	98.3±0.3	48.5±2.2	2.2±0.2
1.0 mM	97.8±0.8	24.2±2.3*	2.0±0.3
TMIOH.HCl			
0.1 mM	99.2±0.2	55.5±1.5	2.4±0.2
0.5 mM	99.3±0.1	49.9±2.2	2.0±0.3
1.0 mM	98.0±0.1	29.6±2.1*	1.9±0.4
CTMIO			
0.1 mM	99.2±0.1	65.2±1.5	2.8±0.1
0.5 mM	99.5±0.2	62.4±2.4	2.7±0.2
1.0 mM	99.2±0.2	57.3±3.2	2.0±0.3
QATMIO			
0.1 mM	99.5±0.2	64.5±3.0	2.8±0.1
0.5 mM	98.9±0.4	62.3±2.4	2.6±0.1
1.0 mM	97.5±1.0	57.8±2.0	3.1±0.5

* Mean ± S.E., $n = 4–11$, vs. control, $P < 0.05$.

36]. A vast amount of data on nitroxide reduction is available, but data on oxidation of hydroxylamines is limited. We investigated the oxidation of the hydroxylamine derivative of TMIO in three different cell concentrations ($5 \cdot 10^6$, $1 \cdot 10^7$, $2.5 \cdot 10^7$ cells/ml) and the reduction of the resulting TMIO under 21 and 0% perfused O_2. It was seen that the intersection of the linear regression for k_1 was not "zero", indicating the oxidation of TMIOH even without cells. Oxidation of TMIOH exposed to 21% O_2 in PBS was consistent with this observation: $k_{anto} = (4.00 \pm 0.46) \cdot 10^5\ s^{-1}$. The rate constants k_1, k_2, and k_3, defined in Sect. 2 (Eqs. (1) and (2)), depended on the concentration of cells in linear fashion $k = k^0$ [cell]. The rate constant per cell were $k_1^0 = (4.2 \pm 0.2) \cdot 10^{-12}\ s^{-1}$, $k_2^0 = (8.8 \pm 2.0) \cdot 10^{-12}\ s^{-1}$ and $k_3^0 = (1.8 \pm 0.1) \cdot 10^{-11}\ s^{-1}$.

3.4 Cytotoxicity of Nitroxides in CHO Cells

Table 2 shows the effect of nitroxides on the cells. None of the nitroxides significantly affected the cellular oxygen consumption rates. The Trypan blue exclusion test did not indicate damaging effects of the nitroxides or the hydroxylamine at 1.0 mM or less. The more stringent clonogenicity assay, however, indicated that TMIO and its corresponding hydroxylamine TMIOH.HCl at 1.0 mM significantly decreased colony formation after long-term exposure.

4 Discussion

The results presented here show that the TMIO family of nitroxides has properties that can be useful for applications in viable systems: sufficient stability and low cytotoxicity. They also appear to have EPR spectral characteristics that are favorable for oximetry, especially the presence of superhyperfine splitting of the lines. At low oxygen concentrations such narrow lines provide oxygen-dependent changes in the line widths that are more easily quantified as compared to small changes in lines with large line widths.

All of the nitroxides showed high bioreduction rates under zero oxygen. This phenomenon is consistent with previous reports in which different nitroxides were investigated [25–32, 36]. TMIO had bioreduction rates similar to those of Tempone and faster than those of the pyrrolidine ring compound, PCA. The high bioreduction of TMIO under zero oxygen may limit its potential application in functional biological systems. The carboxyl-modified isoindoline nitroxide CTMIO had lower bioreduction rates than its parent nitroxide TMIO, suggesting that chemical modification unrelated to the nitroxyl moiety can change the rates of bioreduction of these types of nitroxide and thus may be an effective method to enhance the lifetime of these nitroxides in biological systems.

We included QATMIO in the study with the expectation that such a highly charged hydrophilic nitroxide would not readily cross the cell membrane and therefore would not be reduced rapidly by intact cells [28, 36]. Consistent with

this expectation, QATMIO was resistant to bioreduction as long as the cell membranes were intact. These characteristics of QATMIO may be useful to provide a long-term monitor of extracellular oxygen in cell suspensions or in the vascular system in intact animals.

The apparent rate constants of TMIOH oxidation and reduction of the resulting TMIO increased with cell concentration, but the rate constants/cell did not significantly differ. These kinetics data are useful in understanding the pathways that can be dominant under various biological conditions and can be used to determine oxygen concentration by monitoring hydroxylamine oxidation.

For the use of nitroxides in viable systems, it also is essential to demonstrate that the nitroxides do not interfere with physiological functions of the cells or animal. These nitroxides appear to have the required low cytotoxicity.

In conclusion, the present study indicates that TMIO and its analogues are potentially useful probes for viable systems. They may be especially useful for measurements of concentrations of oxygen.

Acknowledgements

This study was supported by NIH grant RO1 GM 34250 and used the facilities of the NCRR supported EPR Center for Viable Systems (P41 RR11602).

References

1. Glockner J.F., Swartz H.M., Pals M.: J. Cell. Physiol. **140**, 505–511 (1989)
2. Hu H., Sosnovsky G., Swartz H.M.: Biochim. Biophys. Acta **112**, 161–166 (1992)
3. Glockner J.F., Norby S.W., Swartz H.M.: Magn. Reson. Med. **29**, 12–18 (1992)
4. Kuppusamy P., Shankar R.A., Zweier J.L.: Phys. Med. Biol. **43**, 1837–1844 (1998)
5. Baker J.E., Froncisz W., Kalyanaraman B.: Free Radic. Biol. Med. **22**, 109–115 (1997)
6. Inoue M., Utsumi H., Kirino Y.: Chem. Pharm. Bull. (Tokyo) **42**, 2346–2348 (1994)
7. Gallez B., Mäder K., Swartz H.M.: Magn. Reson. Med. **36**, 694–697 (1996)
8. Lai C.S., Hopwood L.E., Swartz H.M.: Exp. Cell Res. **130**, 437–442 (1980)
9. Edgcomb M.R., Sirimanne S., Wilkinson B.J., Drouin P., Morse R.D.: Biochim. Biophys. Acta **1463**, 31–42 (2000)
10. Zhang R., Goldstein S., Samuni A.: Free Radic. Biol. Med **26**, 1245–1252 (1999)
11. Spooner P.J.R., Veenhoff L.M., Watts A., Poolman B.: Biochemistry **38**, 9634–9639 (1999)
12. Swartz H.M., Chen K., Hu H.P., Hideg, K.: Magn. Reson. Med. **22**, 372–377 (1991)
13. Sano H., Naruse M., Matsumoto K., Oi T., Utsumi H.: Free Radic. Biol. Med. **28**, 959–969 (2000)
14. Mäder K., Bacic G., Domb A., Elmalak O., Langer R., Swartz H.M.: J. Pharm. Sci. **86**, 126–134 (1997)
15. Offer T., Mohsen M., Samuni A.: Free Radic. Biol. Med. **25**, 832–838 (1998)
16. Krishna M.C., DeGraff W., Hankovszky O.H., Sar C.P., Kalai T., Jeko J., Russo A., Mitchell J.B., Hideg K.: J. Med. Chem. **41**, 3477–3492 (1998)
17. Shankar R.A., Hideg K., Zweier J.L., Kuppusamy P.: J. Pharmacol. Exp. Ther. **292**, 838–845 (2000)
18. Hahn S.M., Krishna M.C., DeLuc A.M., Coffin D., Mitchell J.B.: Free Radic Biol. Med. **28**, 953–958 (2000)
19. Dikalov S., Skatchkov M., Fink B., Bassenge E.: Nitric Oxide **1**, 423–431 (1997)
20. Dikalov S., Skatchkov M., Bassenge E.: Biochem. Biophys. Res. Commun. **231**, 701–704 (1997)

21. Miura Y., Utsumi H., Kashiwagi M., Hamada A.: J. Biochem. (Tokyo) **108**, 516–518 (1990)
22. Lai C.S., Hopwood L.E., Hyde J.S., Lukiewicz S.: Proc. Natl. Acad. Sci. USA **79**, 1166–1170 (1982)
23. Swartz H.: Adv. Exp. Med. Biol. **345**, 799–806 (1994)
24. James P.E., Grinberg O.Y., Michaels G., Swartz H.M.: J. Cell. Physiol. **163**, 241–247 (1995)
25. Kocherginsky N., Swartz H.M.: Nitroxide Spin Labels: Reactions in Biology and Chemistry. Boca Raton: CRC Press 1995.
26. Sentjurc M., Pecar S., Chen K., Wu M., Swartz H.M.: Biochim. Biophys. Acta **1073**, 329–335 (1991)
27. Kroll C., Langner A., Borchert H.H.: Free Radic. Biol. Med. **26**, 850–857 (1999)
28. Suzuki-Nishimura T., Swartz H.M.: Free Radic. Biol. Med. **17**, 473–479 (1994)
29. Chen K., Glockner J.F., Morse P.D. II, Swartz H.M.: Biochemistry **28**, 2496–2501 (1989)
30. Chen K., Morse P.D. II, Swartz H.M.: Biochim. Biophys. Acta **943**, 477–484 (1988)
31. Chen K., Swartz H.M.: Biochim. Biophys. Acta **970**, 270–277 (1988)
32. Morse P.D. II, Ruuge E.K., Petro M.J., Swartz H.M.: Biochim. Biophys. Acta **1034**, 298–302 (1990)
33. Reid D.A., Bottle S.E., Micallef A.S.: Chem. Commun. **17**, 1907–1908 (1998)
34. Micallef A.S., Bott R.C., Bottle S.E., Smith G., White J.M., Matsuda K., Iwamura H.: J. Chem. Soc. Perkin Trans. **2**, 65–71 (1999)
35. Micallef A.S., Bottle S.E., Gillies D.G., Hughes D.S., Sutcliffe L.H.: J. Chem. Soc. Perkin Trans. (2001) in press.
36. Swartz H.M., Sentjurc M., Morse P.D. II: Biochim. Biophys. Acta **888**, 82–90 (1986)
37. Iannone A., Hu H., Tomasi A., Vannini V., Swartz H.M.: Biochim. Biophys. Acta **991**, 90–96 (1989)

Authors' address: Harold M. Swartz, EPR Center, Department of Diagnostic Radiology, Dartmouth Medical School, Hanover, NH 03755, USA
E-mail: harold.swartz@dartmouth.edu