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Esterified Trityl Radicals as Intracellular Oxygen Probes

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Abstract

Triarylmethyl (trityl) radicals exhibit high stability and narrow line width at physiological conditions which provide high sensitivity and resolution for the measurement of O₂ concentration, making them attractive as EPR oximetry probes. However, the application of previously available compounds has been limited by their poor intracellular permeability. We recently reported the synthesis and characterization of esterified trityl radicals as potential intracellular EPR probes and their oxygen sensitivity, redox properties and enzyme-mediated hydrolysis were investigated. In this paper, we report the cellular permeability and stability of these trityls in the presence of bovine aortic endothelial cells. Results show that the acetoxymethoxycarbonyl-containing trityl AMT-02 exhibits high stability in the presence of cells and can be effectively internalized. The intracellular hydrolysis of AMT-02 to the carboxylate form of the trityl (CT-03) was also observed. In addition, this internalized trityl probe was applied to measure intracellular O₂ concentrations and the effects of menadione and KCN on the rates of O₂ consumption in endothelial cells. This study demonstrates that these esterified trityl radicals can function as effective EPR oximetry probes measuring intracellular O₂ concentration and consumption.

Keywords

stable free radical; electron paramagnetic resonance; sensors; oximetry; redox state; cellular respiration

1. Introduction

Electron paramagnetic resonance (EPR) spectroscopy and imaging have been extensively used to measure physiological parameters such as tissue metabolic activity, redox state, and oxygen (O₂) concentration [1-5]. Significant progress in low-frequency EPR instrumentation has been achieved over the past years for the in vivo detection of free radicals [6-11]. One of the major focuses of EPR spectroscopy and imaging has been to map the spatial distribution of dissolved O₂ in tissue. Owing to its high specificity, sensitivity and non-invasiveness, EPR-based measurement of O₂ concentration, also known as EPR oximetry, shows advantages over other techniques such as the Clark-type electrodes [12], fluorescence [13], ¹⁹F-nuclear magnetic resonance spectroscopy [14], blood O₂ level-dependent imaging [15] and near-infrared

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imaging [16,17]. In principle, since O₂ is paramagnetic, paramagnetic probes can interact with O₂ via *Heisenberg* exchange interaction which results in EPR spectral line broadening of the probes and the magnitude of this broadening can be correlated with O₂ concentration. Over the past two decades, EPR oximetry has evolved as an alternative technique for the accurate and precise determination of O₂ concentration in biological systems including cells and tissues [18-20]. However, the full potential of the technique is still far from being realized due in part to lack of spin probes that are stable, sensitive to O₂, narrow linewidth (ΔB_{pp}), and have target specificity to penetrate and localize within cells.

Although there are a variety of endogenous free radicals present in biological systems [21], they are only present in very low concentrations due to their short half-lives. Thus, exogenous paramagnetic probes have to be introduced into the system under investigation. The spectral response of these probes through various physico-chemical interactions from the redox state of a system is then measured. There are a spectrum of O₂-sensing probes which include particulate-based probes such as lithium phthalocyanine [22], and synthetic char [23], soluble probes such as India ink [24], nitroxides [25], and trityl compounds [26,27]. While particulate probes are suitable for the measurement of pO₂, soluble probes measure dissolved O₂ concentrations. Among the commonly employed probes, nitroxides are the most popular. However, the facile nitroxide reduction in the presence of biological reductants has limited the application of these paramagnetic compounds in vivo. The presence of hyperfine splitting and moderately broad ΔB_{pp} from nitroxides limit the maximum obtainable image resolution.

Recently, a series of substituted forms of triphenylmethyl radicals (trityl radicals) such as OX063 and CT-03 (Figure 1) have been initially developed by Nycomed Innovations (now a subsidiary of GE Healthcare) for use as contrast agents in Overhauser magnetic resonance imaging [28-30]. These sterically crowded trityl radicals show high stability towards various biological oxidoreductants with a single and sharp EPR signal, and therefore are well-suited for EPR imaging applications but their availability remains problematic. Although synthesis of the trityl radicals has always been a challenge in EPR imaging applications, significant progress has been made in our group for large-scale synthesis of the novel hydrophilic trityl CT-03 [30]. Due to the anionic character of CT-03 at physiological pH, it does not enter the cells and therefore the utility of this paramagnetic compound is limited only to extracellular measurements. It is therefore important to develop new trityl radicals as intracellular O₂ probes in order to be able to measure alterations in O₂ metabolism in normal physiology and disease.

An important strategy for introducing the trityl into the cells is to prepare its ester precursor which can cross cell membranes and subsequently be transformed into the carboxylate form by intracellular esterases. This strategy has been successfully used to internalize the nitroxide into different cell lines and increase its retention time in brain cells [31-33]. Moreover, this method is also commonly used in the development of fluorescent intracellular imaging reagents [34].

Recently, we reported [35] the synthesis of several hydrophobic ester-derivatized tetrathiatriarylmethyl radicals AMT-02, AMT-03, BT-03 and MT-03 (Figure 1). Like the charged trityls, these radicals have narrow EPR line widths, high O₂ sensitivity and stability. Moreover, we also demonstrated that the acetoxymethoxycarbonyl-containing trityls AMT-02 and AMT-03 can undergo esterase-mediated hydrolysis to the hydrophilic trityl CT-03. It can therefore be expected that these hydrophobic trityls are able to cross the cell membrane, and upon hydrolysis by intracellular esterases, CT-03 can be formed and retained intracellularly. This strategy may be effective for transporting CT-03 into cells and for measurement of the intracellular O₂ concentrations.

Herein, we investigated the cell permeability of the ester-derivatized trityl radicals and compared their decay kinetics in the presence of bovine aortic endothelial cells (BAECs). The intracellular hydrolysis of AMT-02 and distribution of the resulting EPR-active components as well as the O₂ consumption rates of CT-03-loaded BAECs are presented.

Materials and Methods

Preparation of solutions

The ester-derivatized trityl radicals, AMT-02, AMT-03, BT-03, MT-03 and hydrophilic trityl CT-03 were synthesized as described in the literature [35]. Stock solutions of ester-derivatized trityl radicals, AMT-02, AMT-03, BT-03 and MT-03 (1 mM) were prepared in dimethyl sulfoxide (DMSO). In the case of CT-03, the stock solution (1 mM) was prepared in phosphate buffer (0.2 M, pH 7.4). Before the cellular experiments were conducted, the stock solution was diluted 10-fold with phosphate buffered saline (PBS) and added to the cell culture media. In order to prevent the precipitation of the trityls, the final media contained 5% (v/v) of DMSO.

EPR spectroscopy

All EPR spectra were recorded at room temperature using a Bruker X-band EPR spectrometer with the following parameters: microwave power = 2 mW; center field = 3514 G; modulation frequency = 100 kHz; Scan time = 41.84s. Modulation amplitude used was 0.06 G unless otherwise indicated.

Cell culture

BAECs were obtained from Cell Systems (Kirkland, USA). Cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), glutamine (30 µg/ml), penicillin (100 units/ml), streptomycin (100 µg/ml), MEM non-essential amino acids (MEM-NEAA, 1%) and endothelial cell growth supplement (EGS, 10 µg/ml) at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were used between 7 and 10 passages.

Intracellular uptake of the ester-derivatized trityl radicals

BAECs were incubated with the trityl radicals (20 µM) for 1 h at 37 °C, at which point, the medium containing the trityl radicals was removed and the cells were washed three times with PBS. After treatment with trypsin, the suspended cells were centrifuged at 500g for 5 min and washed three times with PBS. The resulting cell pellet was resuspended in PBS at a concentration of 2.0×10^6 cells/mL. Then, the cell sample was transferred to a glass capillary tube and the EPR spectrum was recorded using a modulation amplitude of 0.4 G.

Decay kinetics of the ester-derivatized trityls in the presence of BAECs

BAECs were incubated with the trityl radicals (20 µM) for 1 h at 37 °C, at which point the medium containing the trityl radicals was removed and the cells were washed three times with PBS. After treatment with trypsin, the suspended cells were centrifuged at 1500g for 2 min and washed twice with PBS. The resulting cell pellet was resuspended in DMEM at a concentration of 2.0×10^6 cells/mL. This time point was considered to be $t = 0$ in the Figure 3. The remaining cell suspension was gently agitated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. At 1, 2, 3, 4 and 5 h intervals, aliquots of the cells were taken for EPR analysis using a modulation amplitude of 0.4 G.

Intracellular distribution of AMT-02

Cells were incubated with a solution of AMT-02 (20 µM) for 1 h, and the cells were centrifuged at 500g at 4 °C for 5 min and washed three times with cold PBS. The resulting cell pellet was

resuspended in 900 μL of PBS after treatment. An aliquot of 100 μL of the cell suspension was taken for EPR analysis. The remaining suspension was treated with protease inhibitor (complete, Roche, 100 μL) and lysed by sonolysis. The cell suspension was sonicated for 5 seconds in an ice bath. The process was repeated 8 times at 1 min interval. After the sonolysis, 100 μL aliquots of cell lysate were taken for EPR analysis. The rest of the suspension was centrifuged at 15000g for 20 min at 4 $^{\circ}\text{C}$. The resulting cell lysate pellet was resuspended in 800 μL of PBS and analyzed by EPR. The supernatant was immediately filtered using a centrifugal filter device (Amicon ultra-4, 5000MWCO) to separate the biomolecules with molecular weight higher than 5 kD from the supernatant. Finally, the filtrate and the >5 kD macromolecular fraction (resuspended in 800 μL of PBS) were analyzed by EPR.

Binding of CT-03 with bovine serum albumin

CT-03 (20 μM) was mixed with bovine serum albumin (BSA) (100 μM) in PBS. After 10 min, 500 μL of the solution was filtered using a centrifugal filter device to separate CT-03 bound to the protein from free CT-03. The EPR analysis was carried out on the filtrate as well as the resuspended protein retentate in 500 μL of PBS.

Time-dependent intracellular hydrolysis of AMT-02 in BAECs

BAECs were incubated with AMT-02 solution (20 μM) for 1 h at 37 $^{\circ}\text{C}$, at which point the medium containing AMT-02 was removed and the cells were washed three times with PBS. The cells were then trypsinized, suspended and centrifuged at 500g for 5 min. The resulting cell pellets were washed twice with PBS and resuspended in DMEM at a concentration of 2.5×10^6 cells/mL. An aliquot of the cell sample was transferred to a 50 μL capillary tube and the EPR spectrum recorded at room temperature. This time point is $t = 0$ in Figure 6. The remaining cell suspension was gently agitated at 37 $^{\circ}\text{C}$ in a humidified atmosphere of 5% CO_2 and 95% air. 50 μL aliquots of the cell suspension were then taken for EPR analysis at 1, 2, 3, 4 and 5 h time points.

Effect of probenecid on the intracellular retention of the trityl radical

Cells were pelleted at 500g at 4 $^{\circ}\text{C}$ for 5 min and washed three times with cold PBS after incubation with a solution of AMT-02 (20 μM) for 1 h. The resulting pellet was resuspended with DMEM at a concentration of 2×10^6 cells/mL in the absence and presence of probenecid (1 or 5 mM). An aliquot of the cell suspension (100 μL) was transferred to a capillary tube, which was analyzed for EPR. This time point is $t = 0$ in Figure 7. The remaining cell suspension was gently agitated at 37 $^{\circ}\text{C}$ in a humidified atmosphere of 5% CO_2 and 95% air at different time durations. Thereafter, an aliquot of the cell suspension was taken and centrifuged at 500g at 4 $^{\circ}\text{C}$ for 5 min. The supernatant was taken for EPR analysis and the resulting cell pellet was washed three times with cold PBS and EPR analysis was carried out.

O₂ sensitivity of the intracellular CT-03

As described above, the trityl-loaded cells (2×10^7 cells/mL) were continuously incubated for 3 h with gentle agitation at 37 $^{\circ}\text{C}$ in the presence of probenecid (5 mM). Potassium cyanide (200 μM) was then added to stop cellular respiration without changing the environment of the trityl in the cells. The resulting cell suspensions were transferred into a gas-permeable Teflon tube (i.d. = 0.8 mm) and the tube was then sealed at both ends. The sealed sample was placed inside a quartz EPR tube with open ends. Pure N_2 or N_2/O_2 mixture with varying concentrations of O_2 was allowed to pass into the EPR tube. EPR spectra were continuously recorded until the peak-peak linewidths remained constant. The O_2 sensitivity of the intracellular CT-03 (1.00 ± 0.03 mG/mmHg) was obtained by examining the variation of the linewidth as a function of the O_2 concentration.

Measurement of O₂ consumption of BAECs

As described above, the trityl-loaded cells (8×10^6 cells/mL) were continuously incubated for 3 h with gentle agitation at 37 °C in the presence of probenecid (5 mM). Then, the cell suspensions were drawn into a capillary tube (final volume 85 μ L) and both ends of the tube were immediately sealed. Care was taken to avoid entrapment of any air bubble inside the capillary. EPR measurements of the O₂ consumption were immediately performed. Menadione (25 μ M) or KCN (100 μ M) were also added to investigate their effects on O₂ consumption by BAECs. The following expression was used to calculate the O₂ concentration as expressed in nanomole per mL: $c = (LW - LW_0) \cdot \alpha / S$, where c is O₂ concentration, LW is linewidth at designated O₂ concentration, LW_0 is linewidth at anaerobic condition (120 mG), α is the solubility of O₂ in water (1.59 nmol/mmHg/mL at 22 °C) and S is the O₂ sensitivity of intracellular CT-03 (1.00 ± 0.03 mG/mmHg).

Results

Intracellular uptake of the ester-derivatized trityl radicals

In order to accurately determine the intracellular O₂ concentration, the probe must be taken up by the cells at a sufficient concentration. It is therefore important to investigate the cell permeability of various trityl radicals. For this purpose, BAECs were incubated with different trityl radicals at 20 μ M at 37 °C for 1 h. After extensive washing, the cells were resuspended, and then EPR spectra were recorded to quantitatively determine the intracellular uptake of the trityls. As shown in Figure 2, the single peak EPR signal could be observed in cell suspensions for various ester-derivatized trityls, whereas no EPR signal was observed for the hydrophilic tricarboxylated trityl, CT-03, at pH 7.4. Due to the charged nature of CT-03, it is expected that this trityl is cell impermeable. This demonstrates that the ester-derivatized trityl radicals can be taken up by the BAECs but not the negatively charged CT-03.

As shown in Figure 2, the observed EPR signal intensity and ΔB_{pp} of the internalized trityls were dependent on the type and number of ester groups. For example, the most hydrophobic trityl radical, BT-03, gave a relatively broader signal with the ΔB_{pp} of 7.91 G, probably due to self-aggregation of the radical. As for AMT-03 and MT-03, a moderately intense EPR signal with ΔB_{pp} of 4.29 G and 2.76 G, respectively, were observed. It is noteworthy that an intense and sharp signal was detected in the case of AMT-02. AMT-02, is mono-anionic at physiological pH and could perhaps be amphiphilic in nature such that it can partially dissolve in water and still have considerable hydrophobicity to enter the cells. This amphiphilic property of AMT-02 could facilitate transmembrane transport and subsequent esterase-mediated hydrolysis in the cytosol, providing a sharp signal that can be assigned to CT-03 [35].

For comparison, the EPR spectra of the esterified trityls in the supernatant are also shown in Figure 2. The EPR signals of the supernatant for AMT-03, MT-03 and BT-03 have almost identical ΔB_{pp} compared to those of the cell suspensions except for AMT-02. In the supernatant, the EPR spectrum of AMT-02 consists of a sharp and a broad signal with the ΔB_{pp} of ~ 0.73 and 4.45 G, respectively. As previously suggested [35], these sharp and broad signals could be due to dissolved and undissolved (or aggregated) form of AMT-02, respectively. As for CT-03, a very sharp signal with a ΔB_{pp} of 0.41 G can be observed in the supernatant. Due to the use of high modulation amplitude of 0.4 G, the observed ΔB_{pp} of 0.41 G for CT-03 and 0.73 G for dissolved form of AMT-02 were larger than the previously reported values of 0.19 and 0.35 G for CT-03 and AMT-02, respectively.

Decay kinetics of the ester-derivatized trityls in the presence of BAECs

Although trityl radicals have considerably high stability, they could also be quenched by intracellular biological oxidoreductants as demonstrated in our previous studies [35,36].

Therefore, we investigated the effects of incubation time of each of the trityl radicals in the presence of cells on the EPR signal intensity. After incubation with the trityl radicals for 1 h, BAECs were exhaustively washed, resuspended and maintained in DMEM at 37 °C. Thereafter, aliquots of these cell suspensions were taken for EPR analysis at a particular time interval. Double integration of the EPR signal was employed to quantitate the concentrations of the trityls. As shown in Figure 3, decay kinetics varied with the type of trityl used. AMT-03 and AMT-02 shared similar decay kinetics and after 5 h of incubation, their EPR signals only slightly decreased with 78% and 75%, respectively, of the EPR signal intensities remaining. BT-03 and MT-03 exhibited relatively rapid decay with only 39% and 14%, respectively, remaining. However, negligible signal intensity was observed from CT-03 throughout the experiment even after 5h incubation.

Inherent intracellular linewidth broadening

The *in vitro* enzymatic hydrolysis of AMT-02 suggests that AMT-02 was likely to be transformed to CT-03 upon hydrolysis by PLE [35]. Based on this previous observation, intracellular delivery of AMT-02 is expected to result in similar hydrolysis and can give rise to the hydrophilic CT-03 with a narrow EPR linewidth. However, the observed ΔB_{pp} in cells (1.02 G) was larger than CT-03 alone in DMEM (0.41 G), although it was relatively narrower compared to other trityl radicals. The broad EPR line is possibly due to the superimposition of more than one paramagnetic component. To prove this, the EPR parameters were varied and the spectrum of AMT-02 in the cell suspension (Figure 2) appears to be an asymmetric EPR line (Figure 4a), indicating the presence of several paramagnetic components such as: 1) the non-hydrolyzed AMT-02; 2) partially hydrolyzed product of AMT-02; and 3) CT-03. In order to further gain insight into the intracellular behavior of AMT-02, the AMT-02-treated cells were lysed by sonolysis. The cell lysate (Figure 4b) afforded a similar EPR signal as Figure 4a, but had stronger signal intensity. This suggests that the sonolysis could release part of the trityl radical that was strongly bound to macromolecules or the cell membrane, or promote hydrolysis of AMT-02 resulting in the formation of products with a narrow linewidth under the conditions used. After centrifugation, the cell lysate pellet and the supernatant were obtained. EPR analysis showed that the resuspended pellet has a similar but weaker EPR spectrum than the whole lysate (Figure 4c, b), while the EPR signal of the supernatant alone mainly consisted of a very sharp signal with a ΔB_{pp} of 287 mG (Figure 4d) which is slightly broader than the ΔB_{pp} observed for CT-03 (190 mG) in PBS but closer to the value (230 mG) in the presence of PLE [35]. The cell lysate pellet is primarily composed of membrane components including lipids and proteins. Various kinds of water-soluble small molecules and macromolecular proteins in the supernatant can also cause slight linewidth broadening. Considering that the hydrophilic CT-03 still has considerable solubility in the lipid as evidenced by its partition coefficient of ~ 0.27 at pH 7.4 [30], the signal in the cell lysate pellet could therefore be derived from a mixture of CT-03 and the non or partially hydrolyzed paramagnetic esters mentioned above.

It was previously proposed that the trityl radicals could bind to human serum albumin [37] and that this did not result in a major increase of the ΔB_{pp} . It is expected that the slight line broadening in the supernatant originates from CT-03 bound to intracellular proteins or other biomacromolecules (Figure 4d). Hence, using a centrifugal filter device, we separated most of the biomacromolecules with molecular weight of more than 5 kD from the supernatant. The resulting fraction was redissolved in PBS and the EPR spectrum was obtained as shown in Figure 4e but no EPR signal was obtained from the filtrate (Figure 4f). This result implies that the signal observed in Figure 4d originates mainly from trityl radical(s) bound to biomacromolecules.

In order to further confirm this, we studied the effect of BSA on the ΔB_{pp} of CT-03. As shown in Figure 5a, a ΔB_{pp} of 192 mG was observed in the absence of BSA, while addition of BSA (100 μ M) to CT-03 (20 μ M) led to a ΔB_{pp} broadening of 285 mG (Figure 5b). This linewidth is similar to the values observed from the supernatant (287 mG) and the > 5 kD fraction (287 mG) of the AMT-02-treated cells as shown in Figure 4d and 4e, respectively. Similar to the whole cell studies, when the BSA-CT-03 solution was concentrated by membrane ultrafiltration (5 kD pore size membrane), an EPR signal was observed from the concentrated BSA with ΔB_{pp} of 289 mG but no signal was seen in the protein free ultrafiltrate (Figure 5c,d). Thus, the ΔB_{pp} broadening observed may be due to trityl radical binding to BSA and perhaps other biomolecules.

Intracellular hydrolysis of AMT-02

BAECs were incubated with AMT-02 for 1 h, extensively washed with PBS and resuspended using DMEM medium. The resulting suspension was incubated at 37 °C with gentle shaking and the EPR spectra were recorded at various time intervals. The intracellular hydrolysis of AMT-02 was monitored by observing the increase in the signal intensity of the sharp signal due to CT-03 (Figure 6.A). The hydrolysis was time-dependent and reached a maximum after 3 h of incubation (Figure 6.B). Unlike AMT-02, hydrolysis of the other esterified trityl radicals was not detectable for 5 h possibly due to their lower solubility and/or greater steric hindrance. These results were consistent with our previous observation [35] that the trityl radicals bearing acetoxymethoxyl moiety are more readily hydrolyzed enzymatically.

Effect of membrane anion transporter blockade on the intracellular retention of CT-03

Based on the intracellular hydrolysis of AMT-02, it is of interest to further study the retention of the resulting CT-03 in BAECs. As shown in Figure 7, half-life of CT-03 intracellularly is 0.68 h. At any incubation time, only a sharp signal that is attributable to CT-03 can be observed in the extracellular space (Figure not shown), indicative of the extrusion of CT-03. Due to the anionic nature of CT-03, its extrusion may have a close relationship with the organic anion transporter in endothelial cells [38-40]. Probenecid is an effective pharmacological inhibitor of such ionic transport systems. Therefore, we tested the effect of probenecid on the extrusion of CT-03 and found that the addition of probenecid effectively slowed the intracellular signal decay of CT-03 with half-lives of 1.27 and 1.64 h in the presence of 1 mM and 5 mM probenecid, respectively. These results reveal that the highly charged CT-03 produced by intracellular hydrolysis of AMT-02 is in part extruded, possibly via an organic anion transporter-mediated mechanism.

O₂ consumption measurements

Figure 8 shows variations in O₂ consumption from suspended cells when treated with menadione, an artificial electron acceptor for NADH:quinone oxidoreductase or KCN, a mitochondria inhibitor. EPR measurements of the cell suspensions in a closed tube were performed at various times. In order to avoid efflux of CT-03 after hydrolysis, probenecid (5 mM) was added. Therefore, it can be expected that the measured O₂ concentration is intracellular. In all cases, the initial O₂ concentration is lower than the normal O₂ solubility in water (266 nmol/mL at 22°C) which is due to the lower O₂ concentration in cells or the baseline O₂ consumption by the cells. The untreated cells show a linear decrease of O₂ concentration over time with an O₂ consumption rate (OCR) of 0.58 ± 0.05 nmol/min/ 10^6 cells. The addition of menadione (25 μ M) shows a significant increase in the rate of O₂ consumption with OCR = 0.90 ± 0.08 nmol/min/ 10^6 cells while cells treated with KCN (100 μ M) showed complete inhibition of O₂ consumption.

Discussion

In the present study, our goal was to evaluate the potential application of ester-derivatized trityl radicals as intracellular EPR oximetry and imaging agents. Our results showed that all the ester-derivatized trityl radicals can enter BAECs and consistently afford a singlet EPR signal after 1h incubation with cells, but not the hydrophilic and charged trityl CT-03 (Figure 2). The intracellular ΔB_{pp} of the trityl radicals vary depending on the kind and number of the ester groups. The order of decreasing ΔB_{pp} is: BT-03 (7.91 G) > AMT-03 (4.29 G) > MT-03 (2.76 G) > AMT-02 (1.02 G). Given that these radicals show the ΔB_{pp} of less than 400 mG in DMSO solution under aerobic conditions [35], the broad ΔB_{pp} in the cells could be due to (1) hindered molecular motion due to binding to biomolecules; (2) superimposition of EPR signals of several paramagnetic components; and (3) self-aggregation resulting from low solubility. The fact that addition of 70% glycerol (V/V) to the DMSO solution of the trityls did not lead to significant change in their ΔB_{pp} (data not shown) indicates that the molecular motion of trityl radicals has only minor effect on the ΔB_{pp} , thus excluding the first possibility. The low solubility of AMT-03, BT-03 and MT-03 leads mainly to their self-aggregation and subsequently to line broadening which is independent of modulation amplitude. In contrast, amphiphilic AMT-02 does not exhibit self-aggregation in the cell but the observed inherent line broadening is due to overlapping signals from the formation of several products including CT-03 and incomplete hydrolytic products (Figure 4a).

Stability studies in the presence of BAECs showed that the trityl radicals AMT-02 and AMT-03 containing acetoxymethoxy group exert high stability such that about 75% of the signal intensity still remains after 5h incubation in the presence of cells. In comparison, BT-03 and MT-03 have lower stability. The varying stability of all of trityls could be due to the difference in their redox potentials. Previously [35], we demonstrated that AMT-03 has high oxidation potential of 0.492 V versus Fc^+/Fc in dichloromethane, which is much larger than the values for BT-03 (0.292 V versus Fc^+/Fc) and MT-03 (0.414 V versus Fc^+/Fc) in the same solvent. Although AMT-02 has a low first oxidation potential (0.232 V versus Fc^+/Fc), its second oxidation potential (0.421 V versus Fc^+/Fc) involved in the radical oxidation is relatively higher than the values for BT-03 and MT-03. Several studies also verified that the trityl radicals are sensitive to oxidizing reagents such as O_2 , superoxide and methyl radicals, but are inert to biological reducing reagents such as ascorbate and glutathione [29,41]. The oxidation of trityl radical by superoxide radical has been proposed to be an effective method for the measurement of superoxide in biological systems [36,42-44]. The marked stability of the esterified trityls, especially that of AMT-03 and AMT-02, makes them attractive for in vivo EPR applications.

Considering that AMT-02 has relatively sharp EPR line and is hydrolyzed to the tricarboxylate CT-03, this trityl appears to be more useful as an intracellular EPR oximetry probe than the other esterified trityl radicals evaluated in this study. AMT-02 was observed to be hydrolyzed to CT-03 which has higher water solubility and sharper EPR signal. The EPR spectrum obtained after 1 h incubation of AMT-02 with cells consists of at least two components: a broad signal due to the unhydrolyzed AMT-02 and a sharp signal due to CT-03 (Figure 4a). The correct assignment for CT-03 was verified by the fact that AMT-02 can be easily hydrolyzed to CT-03 by esterases as previously shown [35] and that the hydrolytic product obtained has similar ΔB_{pp} with CT-03 under the studied conditions. Furthermore, intracellular distribution studies of AMT-02 also suggested that the hydrolysis of AMT-02 was incomplete after 1h incubation as demonstrated by the presence of its broad signal in the cell membrane fraction (Figure 4). Increasing incubation time to the AMT-02-loaded cells enhanced the sharp signal intensity due to CT-03 formation (Figure 6). Nearly complete intracellular hydrolysis of AMT-02 was observed as evidenced by the increased amount of CT-03 formed and the complete disappearance of AMT-02 after 3 h of incubation (Figure 6). Since the resulting CT-03 can be in part extruded by the cells possibly via the organic anion transporter (Figure 7), probenecid,

an inhibitor of the organic anion transporter, can effectively extend the intracellular retention of the trityl. Therefore, in the presence of probenecid, AMT-02 is effective as an intracellular probe for extended periods of time. Furthermore, our experiments show that the intracellular CT-03 formed has good O₂ sensitivity. AMT-02 was employed to measure the OCR in BAECs and the value of 0.58 ± 0.05 nmol/min/10⁶ cells (Figure 8) obtained from untreated cells is similar to the previously reported values of 0.87 ± 0.08 nmol/min/10⁶ cells [45] and ~ 0.3 nmol/min/10⁶ cells [46].

In summary, intracellular penetrability of various ester-derivatized trityl radicals has been investigated. All of these trityl radicals are cell permeable and show high stability in the presence of BAECs. While the trityl radicals, AMT-03, BT-03 and MT-03 exhibit broad EPR signals in the cells, AMT-02 affords a relatively sharp signal owing to its intracellular hydrolysis. The hydrolysis of AMT-02 is time-dependent and affords CT-03 as a product with a sharper linewidth but can be in part extruded from cells, possibly via the organic anion transporter. Nevertheless, the use of an inhibitor of organic anion transport such as probenecid, can slow this process and facilitate the use of CT-03 to study intracellular O₂ consumption.

Thus, the esterified trityl radical AMT-02 was found to function as an effective cellular EPR oximetry probe enabling intracellular loading followed by its hydrolysis to the tricarboxylate form CT-03 which has sharp EPR linewidth and is well suited for oximetric measurements. This use of esterified trityl radicals is a promising approach for the measurement of intracellular properties including oxygenation and redox state in cellular and in vivo models.

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CT-03	$R = \text{CH}_3, R_1 = R_2 = R_3 = \text{Na}$
Ox063	$R = (\text{CH}_2)_2\text{OH}, R_1 = R_2 = R_3 = \text{Na}$
MT-03	$R = \text{CH}_3, R_1 = R_2 = R_3 = \text{CH}_3$
BT-03	$R = \text{CH}_3, R_1 = R_2 = R_3 = \textit{tert}\text{-Butyl}$
AMT-03	$R = \text{CH}_3, R_1 = R_2 = R_3 = \text{CH}_2\text{OC}(\text{O})\text{CH}_3$
AMT-02	$R = \text{CH}_3, R_1 = R_2 = \text{CH}_2\text{OC}(\text{O})\text{CH}_3, R_3 = \text{H}$

Figure 1.
Molecular structure of the trityl radicals.

Figure 2.

EPR spectra of trityl radicals (20 μM) in BAEC suspension and their respective ΔB_{pp} after 1 h of incubation. Cells (2×10^6 cells/mL) were washed three times after incubation with PBS and resuspended for EPR analysis at room temperature. See experimental section for the EPR parameters used.

Figure 3.

Decay of the ester-derivatized trityls in the presence of BAECs. After incubation with the trityl radicals for 1 h, cells were extensively washed, resuspended in DMEM and incubated at 37 °C for various times. Aliquots of the cell suspension were taken at 1 h intervals and EPR spectra were recorded at room temperature. Double integration of the EPR signals yielded the relative amount of the trityls. See experimental section for the EPR spectrometric settings used.

Figure 4. EPR spectra of AMT-02 in (a) cell suspension; (b) cell lysate; (c) resuspended cell lysate pellet; (d) supernatant; (e) > 5 kD fraction and (f) filtrate. Details of the preparation of various cellular fractions are described in the Experimental Section.

Figure 5.

EPR spectra of CT-03 in the presence of bovine serum albumin (BSA). (a) CT-03 (20 μ M) in PBS; (b) same as A, but upon addition of BSA (100 μ M); (c) after sample b was concentrated using membrane ultrafiltration (5 kD) and diluted back to the original volume; and (d) filtrate obtained from sample c. The EPR signal in (a) is shown at $\frac{1}{2}$ gain. Modulation amplitude of 0.06 G was used. See experimental section for other EPR parameters.

Figure 6.

(A) EPR spectra of AMT-02 in the presence of BAECs at various incubation times, (a) 0 h; (b) 1 h; (c) 2 h; (d) 3 h. (B) Relative EPR signal intensity as a function of the incubation time. Cells were incubated in the presence of AMT-02 with gentle shaking at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Duplicate EPR measurements were performed on each aliquot of cell suspension at 1h interval.

Figure 7.

Effect of probenecid on the intracellular retention of AMT-02 and its derivatives. The AMT-02-loaded cells were incubated in the absence or presence of probenecid at 37 °C. An aliquot of the cell suspension was taken, centrifuged and the resulting cell pellet was extensively washed and resuspended for EPR analysis.

Figure 8.

(A) Plots of O₂ concentration as a function of time in the presence of BAECs (8×10^6 cells/mL) alone (■); with addition of menadione (25 μ M) (●); or KCN (100 μ M) (▲) using AMT-02. (B) O₂ consumption rates of the cells; Values are means $\pm$ SD of three experiments.