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Detection of Redox Imbalance in Normal Lymphocytes with Induced Mitochondrial Dysfunction – EPR Study

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Abstract. *The present study describes a new approach for direct imaging of redox status in live cells using paramagnetic spin-probes, which allows evaluation of the level of oxidative stress due to overproduction of superoxide. The method is based on redox cycling of cell/mitochondria-penetrating nitroxide radicals (e.g. mito-TEMPO) and their electron-paramagnetic resonance (EPR) contrast, which makes them useful molecular sensors for analysis of redox status and oxidative stress in cells and tissues. Oxidative stress was induced in normal human lymphocytes by treatment with 2-methoxyestradiol and rotenone (ME/Rot) at different concentrations. This combination provokes mitochondrial dysfunction, which is accompanied by overproduction of superoxide. The EPR measurements were performed in dynamics on X-Band spectrometer after addition of mito-TEMPO to cell suspensions. The intensity of the EPR signal in untreated cells decreased significantly, which indicates a conversion of paramagnetic mito-TEMPO to its non-contrast diamagnetic form (hydroxylamine – mito-TEMPOH) due to reduction. In ME/Rot-treated cells, the signal decreased more slowly and to a lower level with increasing the concentration of ME/Rot. These data indicate an induction of oxidative stress in the cells in a concentration-dependent manner. A very good positive correlation between the intensity of EPR signal of mito-TEMPO and the intracellular level of superoxide was found, analyzed by conventional dihydroethidium test ($R=0.9143$, $p<0.001$). In conclusion, our study demonstrated that cell-penetrating paramagnetic spin-probes, such as mito-TEMPO, are valuable tools for EPR imaging of the superoxide level in live cells, as well as for EPR imaging of mitochondrial dysfunction and metabolic activity, accompanied by superoxide imbalance.*

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Over 50 years of experience in free-radical biology and medicine has shown the crucial role of redox signaling in carcinogenesis, neurodegeneration, inflammation, and other pathologies (1-3). The cells and tissues of healthy mammals are characterized by low steady-state levels of oxidizers (*e.g.* reactive oxygen species, ROS) and higher steady-state levels of reducers (*e.g.* endogenous redox pairs: NADH/NAD^+, NADPH/$NADP^+$, $FADH_2$/FAD, reduced/oxidized glutathione, reduced/oxidized ascorbate, *etc.*). It is widely accepted that increasing ROS above a critical level provokes oxidative stress, which results in redox imbalance and triggers pathogenesis (1-3).

Currently, there is no universal methodology for the estimation of the cellular redox status in living cells. The oxidizing and reducing capacity of cells is determined by many parameters (*e.g.* ROS of different types and origin, products of free radical oxidation of biomacromolecules, status of natural non-enzymatic and enzymatic antioxidant systems, status of endogenous redox pairs, *etc.*) (1-5). Each parameter is analyzed separately by different methodologies. The estimation of cellular redox status is based on comparative analysis of one or several of these parameters and the conclusions are usually controversial.

The present study describes a new approach for direct imaging of redox status in isolated live cells, which allows evaluation of the level of oxidative stress due to overproduction of superoxide. The method is based on the redox cycle of cell-penetrating aminoxyl (nitroxide) spin-

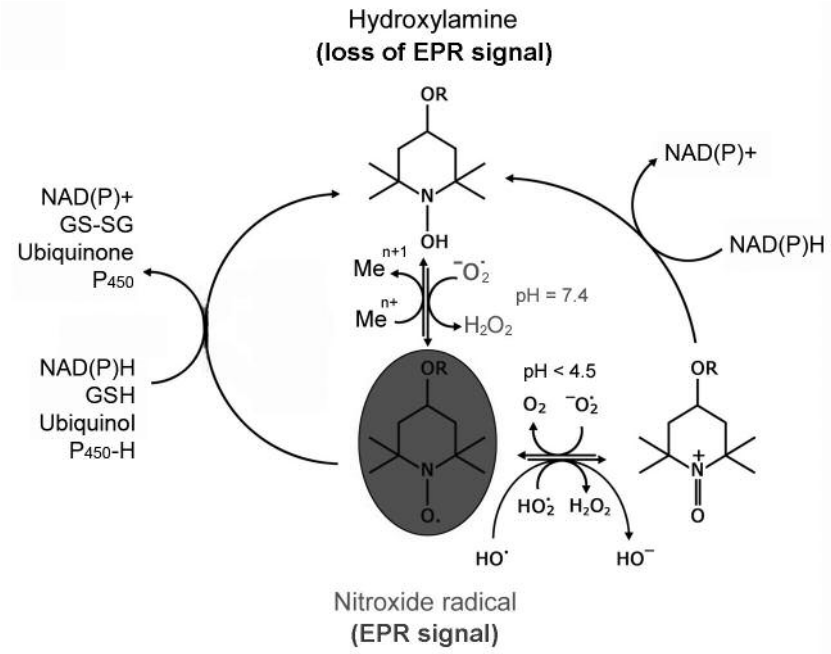

Figure 1. *Redox-cycle of nitroxide spin-probes as a sensing platform for imaging of cellular redox status: principle of the method [according to Zhelev et al. (6)]. EPR: Electron-paramagnetic resonance.*

probes and their electron-paramagnetic resonance (EPR) contrast, which makes them useful molecular sensors for visualization and evaluation of cellular redox balance (6, 7). The paramagnetic nitroxide radicals participate in electron-transfer reactions with oxidizers and reducers, with formation of non-contrast diamagnetic intermediate products (Figure 1). The rate constants of these reactions determine the intensity and dynamics of nitroxide-enhanced EPR signal in living cells.

In vitro studies demonstrated that the nitroxide radical can be converted rapidly to non-contrast diamagnetic forms, hydroxylamine and oxoammonium, by different cellular compounds (free ions of transition metals, superoxide and hydroperoxyl radicals, ubiquinols, NAD(P)H, glutathione, ascorbate, *etc.*) (8-14). Hydroxylamine and oxoammonium are superoxide dismutase (SOD) mimetics and can restore nitroxide radical (10, 12, 15, 16). The interaction of oxoammonium with superoxide occurs very fast (rate constant ~10^9 M^{-1} s^{-1}) (16). However, under physiological conditions, oxoammonium can be reduced effectively to hydroxylamine by NAD(P)H (12). Hydroxylamine also interacts effectively with superoxide at physiological pH~7.4 with recovery of the contrast form (16-18). Therefore, in living cells and tissues, various reducers and oxidizers can be involved (directly or indirectly) in the formation of oxoammonium and hydroxylamine, but only the interaction of hydroxylamine with superoxide can restore the radical form of nitroxide and its EPR contrast. Thus, the dynamics of EPR signals of cell-penetrating nitroxide radicals might serve as a marker of oxidative stress, induced by overproduction of superoxide.

In order to test this assumption, we used an experimental model described by Huang *et al.* (19) and Pelicano *et al.* (20). The authors used rotenone, an inhibitor of mitochondrial complex-I, in combination with 2-methoxyestradiol (ME), an inhibitor of mitochondrial Mn-dependent SOD, to test their effect on cellular superoxide levels by flow cytometry (Figure 2). They found that the combination of ME with rotenone (Me/Rot) caused an accumulation of high amounts of superoxide in cells and strongly minimized the production of hydrogen peroxide.

We applied this experimental strategy to cause overproduction of superoxide and to induce oxidative stress in normal human lymphocytes, as well as to clarify its effect on the dynamics of EPR signals of the nitroxide radical (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (mito-TEMPO) in normal human lymphocytes.

Materials and Methods

Reagents. Mito-TEMPO was purchased from Enzo Life Sciences, Exeter, UK. All chemicals were analytical or HPLC grade. Deionized water (Milli-Q) was used in all model systems.

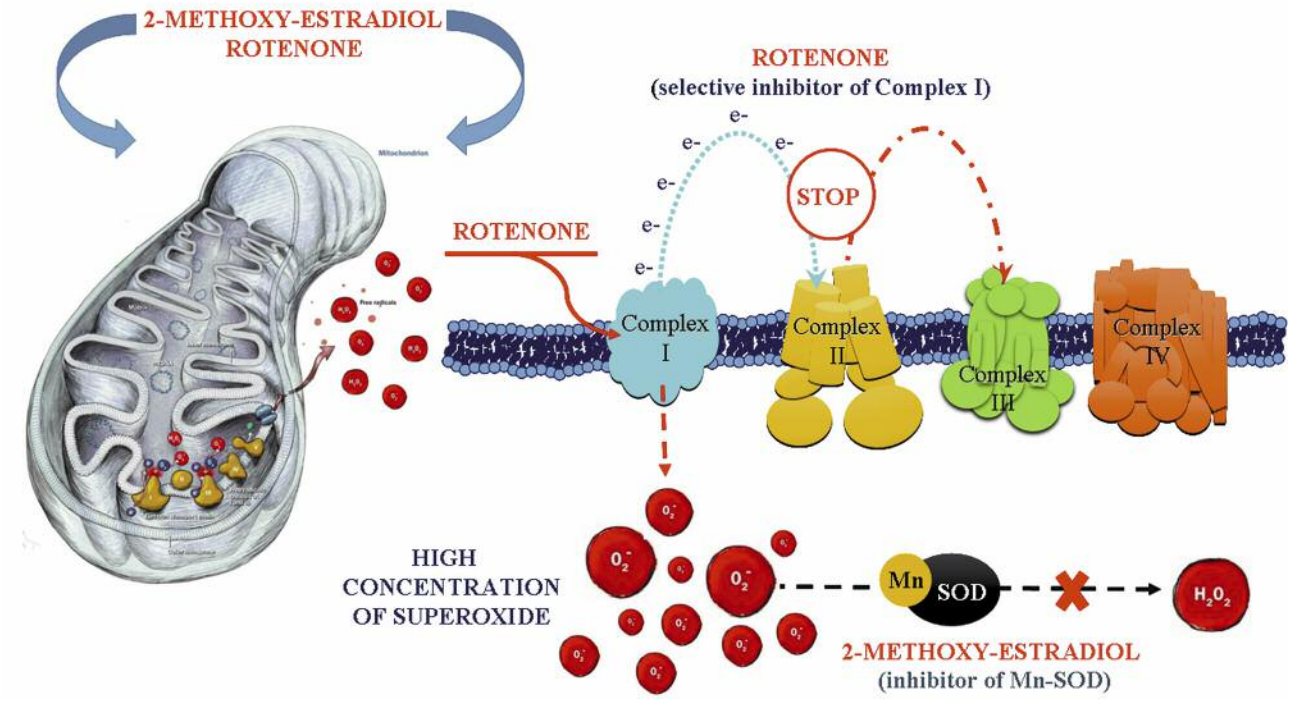

Figure 2. *Biochemical strategy for enhancing superoxide accumulation in cells by inhibition of mitochondrial electron transport chain and mitochondrial superoxide dismutase (Mn-SOD) [according to Huang et al. (19) and Pelicano et al. (20)].*

Cells. Normal human lymphocytes were isolated from heparinized blood of healthy adults (aged 38-40 years) using Lymphosepar-I (Immuno-Biological Laboratories, Takasaki, Japan). The cells were washed five times with phosphate-buffered saline (PBS; 10 mM, pH 7.4) and twice with RPMI-1640 medium, to eliminate traces of transition metals, which can compromise the results. Finally, the cells were re-suspended in the same culture medium and kept in a humidified atmosphere (37˚C, 5% CO_2) for 2 weeks. All experiments were performed within this time interval.

Cell treatment. Twenty-four hours before the experiment, the cells were re-suspended in fresh RPMI-1640 medium. Cell suspension (1.0×10^6 cells/ml) was divided into two parts: non-treated and treated with ME/Rot. Each cell suspension was incubated for 12 h in a cell incubator.

Two different protocols were performed to induce mitochondrial dysfunction: (i) cells were pre-incubated in the absence or presence of ME/Rot (base concentration=300 nM ME and 250 nM rotenone) and then mito-TEMPO (0.1 mM) was added to the cell suspensions; (ii) cells were pre-incubated in the absence or presence of ME/Rot at different concentrations (2-, 5-, 10-, 20-fold the base concentration) and then mito-TEMPO (0.1 mM) was added to the cell suspensions. The incubation was continued at 37˚C in a humidified atmosphere. Aliquots of each cell suspension were collected at different time intervals and EPR measurements were performed at room temperature (20-23˚C).

Before each EPR experiment, the cells were counted and their viability was established by using trypan blue staining and Countess™ automated cell counter (Invitrogen, OR, USA). The cell viability was in the range 92-95%.

EPR measurements. EPR experiments were performed on an X-Band spectrometer (JEOL Inc., Peabody, MA, USA) with standard cavity, at the following parameters: microwave frequency: 9.4 GHz; field strength=336 mT; field modulation frequency=100 kHz; field modulation amplitude=0.063 mT; microwave power: 2.0 mW; time constant=0.01 s; sweep width=10 mT; scan time (sweep time)=1 min, a number of scans: 1. The EPR spectra were integrated and the data were calculated as a percentage from that of the control (0.1 mM of Mito-TEMPO, dissolved in cell-free medium).

Dihydroethidium (DHE) assay. DHE is a cell-penetrating fluorogenic probe, interacting predominantly with superoxide. The DHE assay allows distinguishing between superoxide and hydrogen peroxide (21, 22).

Briefly, DHE was dissolved in dimethyl sulfoxide to 65 mM stock solution (kept at −40˚C), which was diluted with PBS to prepare 50 μM DHE working solution on the day of experiment. Ten microlitres of 50 μM DHA were added to 100 μl of each cell suspension. The samples were incubated for 15 min at room temperature and washed three times with PBS. The fluorescence intensity was detected immediately at λ_{ex}=518 nm and λ_{em}=605 nm, using a microplate reader (TECAN Infinite® M1000; Tecan Austria GmbH, Grodig, Austria).

Results and Discussion

The data in Figure 3 demonstrate the dynamics of EPR signals of mito-TEMPO in untreated and ME/Rot-treated (base concentration) normal human lymphocytes. In

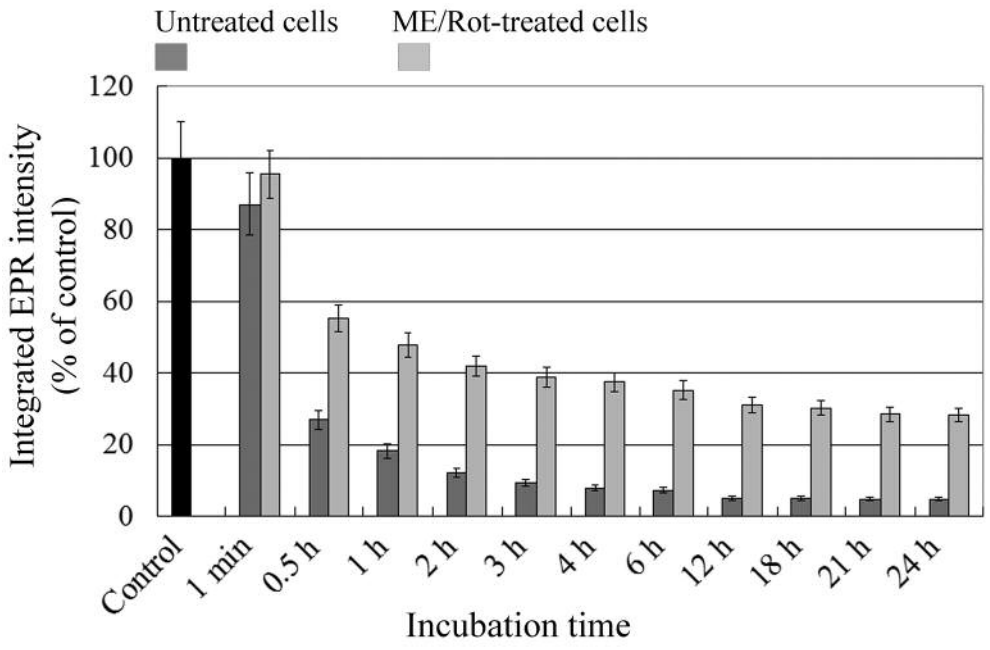

Figure 3. *Dynamics of electron-paramagnetic resonance (EPR) signal of (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (mito-TEMPO) in untreated and 2-methoxy-estradiol (ME)/rotenone (Rot)-treated normal human lymphocytes. Experimental conditions: Cells (1×10^6 cells/ml) were pre-incubated in the absence or presence of ME/Rot (300 nM ME and 250 nM Rot) for 12 h in a humidified atmosphere (5% CO_2, 37˚C). Mito-TEMPO (0.1 mM) was added to the cell suspensions and the incubation was continued under the same conditions. Aliquots of cell suspensions were collected at different time intervals and subjected to EPR analysis. Control sample contained mito-TEMPO (0.1 mM) in cultured (cell-free) medium. The data are mean±SD from four independent experiments.*

untreated cells, the intensity of EPR signal decreased significantly, which indicates a conversion of paramagnetic mito-TEMPO to its non-contrast diamagnetic form (hydroxylamine–mito-TEMPOH) due to reduction. The EPR signal decay is a time-dependent process. The signal decreased rapidly within 1-60 min and very slowly within the next 24 h, reaching a plateau. This may be explained by the gradual penetration of mito-TEMPO through the cellular membrane and its subsequent access to the intracellular reducers and oxidizers. Twenty-four hours after the addition of mito-TEMPO to the cell suspension, the intensity of the signal was only 10% compared to the initial control value (100%). The preliminary treatment of cells with the base concentration of ME/Rot for 12 h influenced the kinetics of EPR signal decay of mito-TEMPO. The signal decreased more slowly, reaching ~30% intensity at 24 h. This indicates an enhancement of the oxidative capacity of the ME/Rot-treated cells in comparison with untreated cells.

Figure 4 shows the dynamics of the EPR signal of mito-TEMPO in normal human lymphocytes, pre-treated with different concentrations of ME/Rot. The signal decreased more slowly and to a lower level with increasing concentration of ME/Rot. At very high concentrations of ME/Rot (10- and 20-fold base concentration), the EPR signal was very similar to that of the control level: 80% (for ME/Rot-treated cells) *versus* 100% intensity (for the control), while at lower concentrations (2- and 5-fold base concentration), it decreased significantly, to about 35% and 60% intensity, respectively. These data indicate that the oxidative capacity of the cells increased with increasing concentration of ME/Rot.

As mentioned above, our experimental model is based on impairment of mitochondrial respiration by ME/Rot, which is accompanied by overproduction of superoxide (18-20). Thus, in order to verify the EPR data, we used a conventional DHE assay to evaluate the level of superoxide in cells treated with different concentrations of ME/Rot. Figure 5A indicates that the level of superoxide increased with increasing concentration of ME/Rot in the cell suspension. A very good positive correlation between the intensity of EPR signal of mito-TEMPO in the cells and the intracellular level of superoxide, analyzed by DHE, was found (Figure 5B).

Superoxide has a pre-eminent role in biology and pathophysiology because it is formed by many mammalian enzymes, has a key role in cell signaling regulation, and serves as a progenitor for the formation of many other ROS, including hydrogen peroxide, peroxynitrite, and lipid

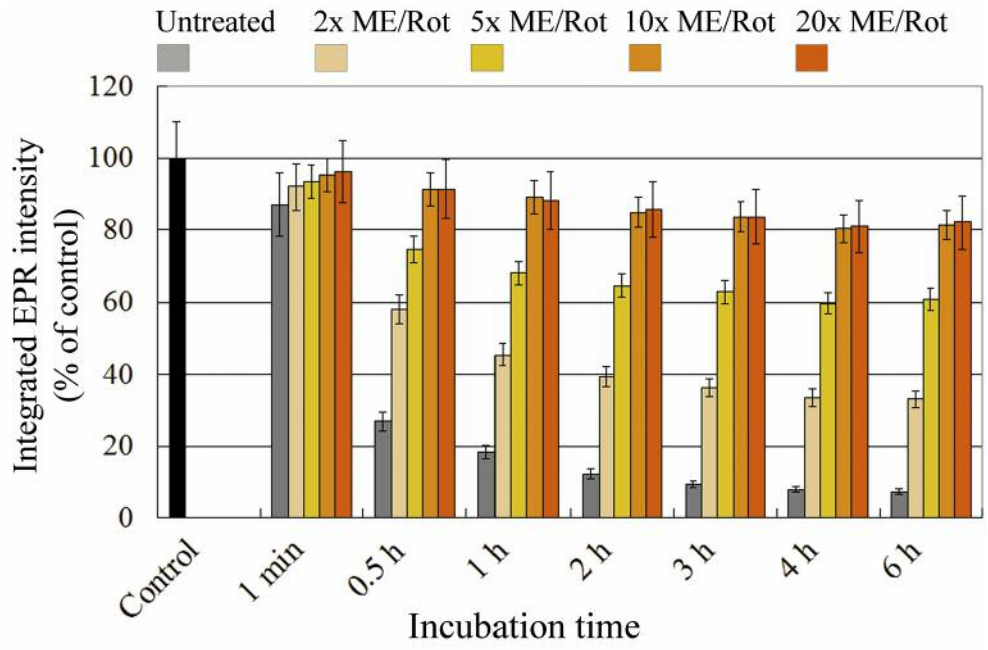

Figure 4. *Dynamics of electron-paramagnetic resonance (EPR) signal of (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (mito-TEMPO) in normal human lymphocytes, treated with different concentrations of 2-methoxy-estradiol (ME)/rotenone (Rot). Cells (1×10^6 cells/ml) were pre-incubated in the absence or presence of 2-, 5-, 10-, and 20-fold base concentration of ME/Rot (=300 nM ME and 250 nM Rot) for 12 h in a humidified atmosphere (5% CO_2, 37˚C). Mito-TEMPO (0.1 mM) was added to the cell suspensions and the incubation was continued under the same conditions. Aliquots of cell suspensions were collected at different time intervals and subjected to EPR analysis. Control sample contained mito-TEMPO (0.1 mM) in cultured (cell-free) medium. The data are mean±SD from four independent experiments.*

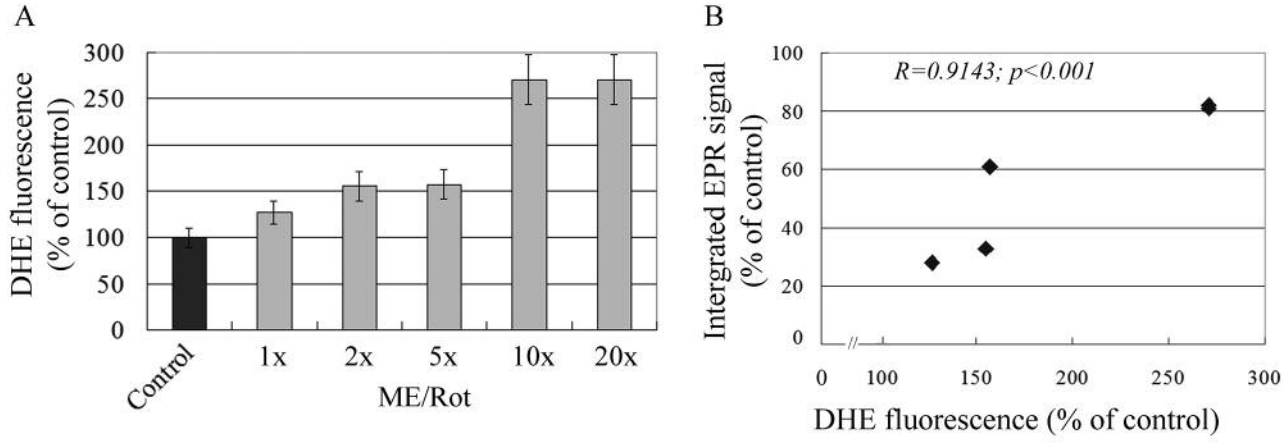

Figure 5. *Level of superoxide (A) and correlation between electron-paramagnetic resonance (EPR) intensity of (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (mito-TEMPO) and superoxide level (B) in normal human lymphocytes treated with different concentrations of 2-methoxy-estradiol (ME)/rotenone (Rot). A: Cells (1×10^6 cells/ml) were pre-incubated in the absence (control) or presence of 1-, 2-, 5-, 10-, and 20-fold base concentration of ME/Rot (=300 nM ME and 250 nM Rot) for 12 h in a humidified atmosphere (5% CO_2, 37˚C). The level of superoxide was analyzed by dihydroethidium (DHE) test. DHE fluorescence in the control was considered 100%. The data are the mean±SD from three independent experiments. B: Cells were treated with different concentrations of ME/Rot (1-, 2-, 5-, 10-, and 20-fold) and the EPR signal intensity was measured 6 h after addition of mito-TEMPO (0.1 mM) to the cell suspensions, when the kinetic curves reached a plateau. R: Correlation coefficient.*

peroxides (1-3, 5). Moreover, overproduction of superoxide is associated with a variety of pathologies and it is a promising molecular marker for their diagnosis, as well as a target for therapy (1-3).

Our study demonstrated that cell-penetrating paramagnetic spin-probes, such a mito-TEMPO, are valuable tools for EPR imaging of superoxide level in isolated live cells, as well as for EPR imaging of mitochondrial dysfunction and metabolic activity accompanied by superoxide imbalance. This spin-probe is characterized by redox cycle, allowing detection of the balance between intracellular oxidizers and reducers (Figure 1). Therefore, mito-TEMPO is a useful tool for EPR imaging of intracellular redox status *in vitro* and *in vivo*.

Recently, Dikalov and Harrison described an excellent overview of the methods for the detection of mitochondrial and cellular ROS (5). Here, we would like to focus only on EPR analysis. One of the earliest methods for detection of superoxide using EPR spectroscopy was spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (23). It is important to distinguish between spin-traps and spin-probes (5). Spin-traps form covalent bonds with ROS, while spin-probes (such as mito-TEMPO) are oxidized by ROS without being bound (24). DMPO and other similar nitrone spin-traps are very useful in studies of isolated biomacromolecules (*e.g.* enzymes) and in pure chemical systems. However, they react with superoxide at very slow rate constants and it is difficult to use them for detection of superoxide in biological systems due to competition with SOD and intracellular reducers (such as ascorbate) (25).

Another nitrone spin-trap, 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO), has been conjugated with a triphenylphosphonium group, which allows a selective penetration into mitochondria (26). Unfortunately, the use of this spin-trap, called mito-DEPMPO, is limited by its slow rate constant for reaction with superoxide, potential toxicity and non-specific effects, and its use in biological systems is therefore unlikely (5, 28, 29). Mito-DEPMPO has to be used at high concentrations (50 mM), which may cause inhibition of mitochondrial respiration due to accumulation of large amounts of lipophilic cations in the mitochondrial matrix and disruption of mitochondrial potential (5, 27).

Dikalov *et al*. reported that cyclic hydroxylamines can be used for measurement of superoxide in cultured cells, tissues, and *in vivo* (30-32). These diamagnetic molecules are oxidized by superoxide and other ROS to form paramagnetic stable EPR-detectable nitroxide radicals with life-times of several hours in cell culture (5, 18, 30-32). Cyclic hydroxylamines react with superoxide much more rapidly then nitrone spin-traps, which enhances the efficiency for detection of intracellular superoxide (32). These diamagnetic probes can be used at relatively low concentrations (0.05-1 mM), minimizing potential toxic side-effects in biological systems.

In this context, our study demonstrated that paramagnetic spin-probes such as mito-TEMPO are also a promising choice for development of EPR methodologies for detection of superoxide, as well as for evaluation of redox status in live cells and tissues.

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