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Low-temperature EPR spectroscopy as a probe-free technique for monitoring oxidants formed in tumor cells and tissues: Implications in drug resistance and OXPHOS-targeted therapies

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Abstract

Oxidants formed from oxidative and nitrative metabolism include reactive oxygen species (ROS) such as superoxide, hydrogen peroxide/lipid hydroperoxides and reactive nitrogen species (RNS) (*e.g.*, peroxynitrite [ONOO⁻] and nitrogen dioxide), and reactive halogenated species (hypochlorous acid [HOCl]). Increasingly, ROS and RNS are implicated in tumorigenesis as well as tumor growth, progression, and metastasis. Recently, ROS were implicated in drug resistance, metabolic reprogramming, and T-cell metabolism in immunotherapy. Mostly, fluorescent probes have been used in cell culture systems. The identity of species is obtained by LC–MS analyses of diagnostic marker products. However, extrapolation of these assays to cancer xenografts is difficult if not impossible. Thus, development of a probe-free assay for monitoring and assessing oxidant formation in tumor cells and tumor xenografts is critical and timely. Here, we describe the use of *ex vivo* electron paramagnetic resonance (EPR) spectroscopy at cryogenic temperatures as a uniquely useful probe-free technique for assessing intracellular oxidation and oxidants via EPR signals from redox centers, particularly iron-sulfur clusters, in mitochondrial and cytosolic redox proteins. Examples of cancer cells subjected to inhibition of mitochondrial oxidative phosphorylation are presented. This *ex vivo* methodology can be readily extended to monitor oxidant formation in tumor tissues isolated from mice and humans.

Keywords

electron paramagnetic resonance; reactive oxygen species; oxidative phosphorylation; mitochondrial targeting

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Role of ROS in cancer biology and immunotherapy

Emerging literature strongly supports the redox signaling implications of reactive oxygen species (ROS) in cancer biology, cancer immunology, and drug resistance (1–6). However, the role of ROS has always been paradoxical in cancer biology (1–6). Small amounts of ROS have been shown to promote tumors, and excessive ROS exert tumoricidal effects (7–10). Tumor cells undergo metabolic reprogramming from oxidative phosphorylation (OXPHOS) to glycolysis or from glycolysis to OXPHOS as in the case of drug-resistant cancer cells. The continued use of antitumor drugs like cisplatin causes resistance to the drug in cancer patients (1–3). A proposed mechanism is the occurrence of metabolic reprogramming in cisplatin-resistant cancer cells (2–4). The increased mitochondrial energy metabolism is linked to enhanced ROS generation in mitochondria as well as increased expression of PGC-1 α , a key translational coactivator promoting mitochondrial biogenesis and biosynthesis of antioxidant machinery (*e.g.*, Nrf2, glutathione synthesis). It was shown that ROS-enhancing agents that are targeted to mitochondria are more effective in overcoming drug resistance (11,12). Mitochondrial sirtuin-3 (Sirt3), an NAD-dependent deacetylase, was reported to regulate the activity of mitochondrial antioxidant enzymes such as manganese superoxide dismutase (MnSOD) and intracellular ROS levels (13,14). Sirt3 is presumably responsible for switching metabolic reprogramming from oxidative phosphorylation back to glycolysis in cancer cells (15). Studies suggest that Sirt3 functions as a tumor suppressor and inhibits ROS formation in cancer cells (16). Alternatively, Sirt3 deficiency enhances ROS formation.

Tumor cells evade immune surveillance by interacting with a surface receptor, programmed cell death (PD-1), through the PD-1 and the ligand, PD-L1. Blocking of PD-1 with the PD-L1 monoclonal antibody (PD-L1 mAb) increased the recognition of tumor cells by T cells leading to increased tumor cell killing (17). The PD-L1 monoclonal antibody enhanced the mitochondrial oxidative metabolism and ROS in cytotoxic T cells (18).

Although PD-1 blockade immunotherapy has vastly changed the landscape of treatment of some cancers (melanoma, lung cancer), many cancer patients still do not favorably respond to immunotherapy (19,20). The lack of response is attributed to an overall exhaustion of T cells caused by mitochondrial dysfunction and decreased energy metabolism. During PD-1 blockade therapy, tumor-reactive CD8⁺ T cells exhibit higher ROS generation (18). It was suggested that mitochondrial stimulation in T cells would enhance its antitumor activity and that unresponsive T cells may be activated by combining PD-1 blockade therapy and mitochondrial ROS stimulators (18). Development of rigorous and artifact-free assays to detect and characterize ROS in tumor cells and tissues is timely and clearly warranted.

Detection of ROS using fluorescence-based redox probes and targeted ROS scavengers

Using redox active fluorescent probes, targeted production of ROS has been proposed as a viable mechanism to overcome drug resistance in cancer cells (3). Confocal fluorescence microscopy was used to measure ROS in several cancer biological applications including cancer drug resistance and immunotherapy (4,18). As discussed in a recent article that

illustrates the potential pitfalls of using fluorescent probes (21), most fluorescent, bioluminescent, and chemiluminescent redox active probes undergo one-electron oxidation to a probe-derived radical intermediate that can reduce oxygen to superoxide and subsequently hydrogen peroxide. This artifactual generation of ROS is probably one of the major reasons for many of the discrepancies. Some notorious probes in this category are dichlorodihydrofluorescein (DCFH), lucigenin, and luminol. The artifactual generation of ROS (*e.g.*, hydrogen peroxide) potentially is responsible for the nonlinear relationship between fluorescence intensity and ROS levels using the DCFH probe (22–25).

In contrast with the probes discussed above, phenanthridinium-based fluorescent probes such as hydroethidine (HE) and Mito-SOX (mitochondria-targeted HE analog) are oxidized to an intermediate radical ($\text{HE}^{\bullet+}$) that does not react appreciably with oxygen to form superoxide. DCFH and HE or Mito-SOX likely are oxidized by a similar oxidant to form a probe-derived radical species via one-electron oxidation. However, the major differentiating factor is the rapid reaction of the HE-derived radical and Mito-SOX-derived radical with superoxide to form the highly characteristic 2-hydroxyethidium (2-OH-E^+) and 2-hydroxy-mitoethidium (2-OH-Mito-E^+) products that can be unambiguously detected and quantified by an HPLC or LC–MS-based technique. Mitochondria-derived ROS (or mROS) are routinely detected using Mito-SOX-derived red fluorescence. Attributing the increased red fluorescence derived from Mito-SOX to mitochondrial superoxide is flawed because the two-electron oxidation product Mito-E^+ also exhibits red fluorescence. Advanced spectral imaging techniques are necessary to differentiate between Mito-E^+ and 2-OH-Mito-E^+ . Thus, 2-OH-Mito-E^+ should be separated and identified by LC–MS for precise detection of mitochondrial superoxide (26–31).

Mitochondria-targeted nitroxides were used as superoxide dismutase mimetics, and results showed that aconitase inactivation in endothelial cells treated with hydrogen peroxide was inhibited by mitochondria-targeted carboxy proxyl (Mito-CP) (32). Similarly, Mito-Tempol has been used as a mitochondria-targeted superoxide dismutase mimetic to inhibit mitochondria-generated ROS and oxidative damage. In these studies, experimental control included the untargeted nitroxide (either CP or Tempol) and the triphenylmethyl cation (32). Although nitroxides conjugated to the triphenylphosphonium moiety elicited the expected results, it is essential to point out a few critical problems in data interpretation. We believe that the most appropriate control is the use of a compound where the nitroxide group is replaced by an acetamido group. For example, the control for Mito-CP was Mito-CP-Ac (33). Mito-CP-Ac does not exhibit the superoxide dismutating ability, but is taken up by cells and accumulates in mitochondria, to a similar extent as Mito-CP (33). Much to our surprise, we found that Mito-CP-Ac inhibited cancer cell proliferation nearly as effectively as did Mito-CP (33). Thus, we revised our interpretation with regard to the role of mitochondrial superoxide in stimulating the proliferation of cancer cells (31,33). The antiproliferative effects of Mito-CP and Mito-CP-Ac are believed to be linked to their abilities to induce mitochondrial redox signaling and not to dismutation of superoxide (33). Based on this work (33), we suggest that investigators using Mito-Tempo as a superoxide dismutation mimetic should use the corresponding Mito-Tempo-Ac that is devoid of superoxide dismutating ability to fully confirm the mechanistic implication of superoxide. At higher concentrations, nitroxides are able to physically quench the fluorescence derived

from redox probes, and this should be taken into consideration as well by measuring ROS-derived specific products formed from the fluorogenic or non-fluorogenic small molecule probes.

This review does not address several related EPR techniques including the use of spin traps and spin probes in the detection of ROS and nitric oxide (NO) in biological systems. These aspects are comprehensively addressed in several recent reviews on EPR (24,34–36). However, we will address here how the spin trapping technique can provide important mechanistic details on the reaction between oxidants (H_2O_2 and ONOO^-) and boronate-based ROS probes (37–39). Whereas H_2O_2 reacts with boronate probes slowly and stoichiometrically with phenolic and aliphatic boronates (37,40), ONOO^- reacts with boronates very rapidly, yielding the same two-electron oxidation phenol (as with H_2O_2) as a major product and nitrated and cyclized minor products that are highly diagnostic for ONOO^- (37,41). The intermediate phenyl radical involved in the minor reaction pathway was detected using the spin trap, 2-methyl-2-nitrosopropane (38). Spin trapping also provided evidence for the intermediacy of a phenyl radical during the oxidation of a boronate-based pro-bioluminescent probe by ONOO^- .

EPR detection of biomarkers of mitochondrial oxidation

Electron paramagnetic resonance (EPR) spectra of mammalian cells and tissues at cryogenic temperatures (5–40 K) are dominated by a collection of signals due to iron-sulfur clusters and ferrihemes of the mitochondrial respiratory chain complexes I–IV, and a $[\text{3Fe4S}]^+$ cluster in aconitase species (42–45). Additional signals due to catalase ferriheme, transferrin non-heme Fe^{3+} , and Mn^{2+} may also be observed under certain experimental conditions. EPR analysis of the frozen material provides a snapshot of the electron distribution along the functional (or dysfunctional) mitochondrial electron transport chain; EPR additionally reports on oxidative damage to redox centers via new or altered EPR signals, reports on expression levels of stress-induced redox proteins, and directly detects stable free radical products of oxidative stress. Recent applications have included mitochondrial disease (44), cancer tissue and cells (21,31), neuromuscular disease (46), Parkinsonism (47), and cardiovascular disease (48).

EPR signals from tissue and eukaryotic cells.

The manifold low-temperature EPR signals from tissue or cells have largely been assigned from studies of isolated proteins, modified or truncated proteins, protein complexes, and mitochondria (43,49). Complex IV (cytochrome *c* oxidase) gives rise to assignable resonances at $g' \sim 3.0$ and $g' \sim 2.2$ due to low-spin heme *a*, and, under some conditions, resonances at $g \sim 2.2$ and 2.0 due to the dinuclear $S = 1/2$ Cu_A center (50,51). Additional complex IV signals at $g' \sim 12$ and 2.95 can be difficult to detect in some tissues and cells due to low signal levels and overlap with the heme *a* $g' \sim 3.0$ resonance, respectively, and are associated with the heme a_3 - Cu_B coupled center (52–54).

Complex (II) (succinate dehydrogenase) gives rise to overlapping signals due to reduced $[\text{2Fe2S}]^+$ (S1) and $[\text{4Fe4S}]^+$ (S2) clusters; the g -values for these signals are dependent on the extent of reduction and the temperature, as the influence of the spin-spin interaction is

dependent on both, but both give rise to an intense derivative feature at $g \sim 1.92$ and contribute to that characteristic signal in the spectra of tissue and cells (55–57). An additional complex II signal due to an oxidized $[3\text{Fe}_4\text{S}]^+$ (S3) signal overlaps extensively, at $g \sim 2.02$, with that from oxidatively deactivated cytosolic aconitase in which the labile Fe_a atom is lost from the active $[4\text{Fe}_4\text{S}]^{2+}$ cluster to form an $[3\text{Fe}_4\text{S}]^+$ cluster (45); these signals can be readily deconvoluted, however, due to their significantly different temperature dependences, *i.e.*, the aconitase signal is extinguished at 40 K and higher temperatures, allowing independent quantitation of S3 (44). An apparent splitting of the S3 $g \sim 2.015$ turning point in the spectrum from beef heart submitochondrial particles and evident as flanking resonances at $g \sim 2.03$ and 1.98 was resolved as being due to the presence of a stable, dipolar-coupled ubisemiquinone pair in the vicinity of S3 in complex II (58).

Of the mitochondrial electron transport complexes, complex III (cytochrome bc_1 complex; CoQH_2 -cytochrome c reductase) is perhaps the least informative in the EPR spectra of tissue and cells. The only signal routinely assignable to complex III is the reduced $[2\text{Fe}_2\text{S}]^+$ Rieske cluster with a distinct sharp g_1 resonance at 2.03, a derivative feature at 1.90 (generally not resolved from the composite ' $g = 1.92$ ' signal), and a very broad g_3 feature at $g \sim 1.78$ that is observable in heart and muscle samples that do not exhibit Mn(II) signals (see, for example, Fig. 5 of (45)) (42,59,60). The other EPR signals observable from isolated complex III are resonances that overlap in the region $g' \sim 3.8$ –3.3 and are due to g_1 of cytochromes c_I , b_L , and b_H (42,61). These signals are not detected in most tissues and will not be discussed further.

Finally, a number of iron sulfur signals are detectable from complex I (NADH:quinone oxidoreductase). There has been some controversy over the assignment of EPR signals of FeS clusters in complex I to structurally characterized ones. A detailed metanalysis of assignments of redox midpoint potentials highlighted how the situation is complicated by a large number of albeit fairly weak magnetic interactions between the clusters (62). In addition, the EPR spectra of many of the clusters not only overlap with each other but also with other signals in the crowded $g \sim 2$ region (63,64). Our approach has been to adopt the assignments of Ohnishi and Nakamaru-Ogiso, also considering the more recent redox analysis of Stuchebrukhov, and applying Occam's razor by using only four nominal FeS clusters to describe the contribution of complex I to the EPR spectrum; in the case of computer fitting (44), this reduces the number of fitting parameters to avoid overfitting (*i.e.*, number of variables > number of observables) the experimental spectrum with an unreasonably large number of highly correlated parameters. Two of the species, N4 and N3, actually exhibit well-resolved g_3 resonances at $g = 1.88$ and 1.86, respectively, and these are also the clusters with the lowest midpoint potentials that can nevertheless be accessed by an NADH^+/NAD couple-determined overall redox potential. The rest of the contribution of complex I FeS centers is considered to be due to the N1b $[2\text{Fe}_2\text{S}]^+$ and N2 $[4\text{Fe}_4\text{S}]^+$ clusters that have midpoint potentials around -240 mV and -270 mV, respectively, and exhibit essentially axial EPR spectra with $g_{\perp} \sim 1.92$. Computer fitting of spectra of tissue have been successful using this limited model (44).

Additional signals in the spectrum arise from Mn(II) of enzymatic or indeterminate origin, rhombic high-spin heme signals from catalase with $g_x \sim 6.45$ and $g_y \sim 5.33$ (49,65), and a

signal at $g \sim 4.2$, with a characteristic splitting at the crossover, due to transferrin Fe^{3+} (66–68). Other signals from Cu^{2+} , Fe^{3+} , and Mn^{2+} due to tissue-specific enzymes may also be present but are not discussed further.

EPR technical considerations.

Some technical aspects should be considered. Cryogenic temperatures for EPR are required, not only to maintain the samples frozen but also because many of the paramagnetic centers are fast relaxing and may have thermally accessible excited spin states; at higher temperatures, their spectra are either undetectable or highly distorted. Different signals have different temperature dependencies and any one temperature represents a compromise that must be accounted for in quantitation. However, the use of two or more temperatures has proven useful in deconvoluting overlapping spectra (44). Mn(II) can be particularly bothersome in spectra of liver tissue and of cultured cells, because the signal overlaps many useful features and contains both absorption- and derivative-like features due to rapid passage that is unavoidable under conditions where the faster relaxing signals can be observed without distortion. Spin concentrations of the individual species are low (generally 1–10 μM), and the overall spectral envelope at X-band is around 5000 G. Therefore, a high-Q resonator is desirable. Spectrometer and environmental stability are needed for long data acquisition times and to minimize baseline artifacts. Microwave frequency stability benefits from stable ambient temperature and humidity, and field-sweep reproducibility (digital field controller; delay to account for hysteresis), electrical stability and RF isolation, and lack of vibration are important. EPR signal amplitudes scale as $(\text{line width})^{-2}$ and can differ by orders of magnitude in the spectra of tissue and cells, and a high-resolution (*e.g.*, 24 bit) signal channel is therefore desirable.

Sample preparation.

The goal of sample preparation is to provide frozen samples that reflect the electron distribution within the electron transport chain, and the chemical identity of ROS-sensitive species, in the intact cell or tissue immediately prior to harvesting. It is key, then, that tissue is excised and frozen before either the exhaustion of reducing equivalents or of a terminal electron acceptor (usually oxygen) alters the local and global redox potentials, and before further non-physiological ROS-mediated damage occurs. EPR spectra of formalin-fixed brain tissue, for example (69), are devoid of almost all of the characteristic signals observed in freshly frozen brain tissue (47,70). Preliminary studies indicate that tissue samples frozen < 3 min after being harvested from freshly sacrificed animals meet these criteria. This places tight constraints on clinical technicians collecting human tissue samples for EPR but is nonetheless achievable in a suitably designed clinical suite with trained staff. Rapid freeze-quenching of viable, intact cells or tissue abruptly halts both molecular diffusion and electron transport between disconnected redox centers, and samples prepared as described here have been found to be stable with respect to their EPR signals for at least six months upon storage at -80°C . Samples are most readily prepared by transfer of fresh tissue into an EPR tube followed by freezing in dry-ice/methanol, liquid nitrogen-chilled isopentane, or liquid nitrogen; the latter is a slower freezing method but is more convenient and safer in a clinical environment due to the lack of flammability. Rapid transfer is effected by extrusion into a hollow tube using a syringe. Ideally, samples should completely fill the volume of the

tube that occupies the active length of the EPR resonator for reproducible quantitation. Smaller samples can be mounted in the center of the active region of the resonator by first freezing a platform of a water-glycerol mixture in the bottom of the tube, adding the tissue, and adding more water-glycerol to the desired height before freezing. The latter step maintains a more-or-less uniform dielectric constant along the active region of the resonator, which maintains consistency of B_1 across the sample and between samples for a given microwave power and resonator. Finally, freeze-thawing of samples has been found to drastically alter the EPR signal, in some cases after a single freeze-thaw cycle, presumably due to ice-crystal disruption and consequent depolarization of the mitochondrial membranes and extinction of the proton gradient. Thawing of frozen samples should, therefore, be avoided at all costs, and thawed samples should be excluded from further study by EPR.

Application to cancer.

Three key pieces of information relevant to cancer cell metabolism and ROS can be gleaned from EPR: metabolic potential, the chemical potential of ROS, and compensatory changes in antioxidant enzymes in response to chronic exposure to ROS and ROS-mediated oxidation.

(i) Information from EPR on the metabolic capacity of the mitochondrion is provided primarily by the measurement of three signals. A sharp distinct signal observed at $g = 1.92$ is largely due to the $g(x,y)$ features of complex I reduced FeS clusters N1b and N2, with additional contributions from $g(y)$ of N3 and N4 and from $g(x,y)$ of complex II S1 and S2. The dominant N1b and N2 clusters exhibit midpoint potentials of -240 mV and -270 mV, respectively (62), and diminution of the $g = 1.92$ signal compared with healthy cells or tissue therefore represents a significant departure of the redox potential from the expected -320 mV, dictated by the NAD^+/NADH couple (71). Any electrochemical potential differential between the mitochondrion and the NAD^+/NADH couple represents a decrease in the potential energy available for ATP generation via proton translocation through complex V. More sensitive biomarkers for redox potential are the N4 ($g(x) = 1.88$; $E_m = -280$ mV) and N3 ($g(x) = 1.86$; $E_m = -325$ mV) clusters, with the isolated $g(x)$ features just upfield of the $g = 1.92$ signal. Unfortunately, with cells and some tissues (*e.g.*, liver), the maximally discriminating N3 feature overlaps with the often more-intense high-field $m_I = -5/2$ resonance of the $m_S = 1/2$ manifold of $S = 5/2$ Mn^{2+} (21,44).

(ii) The chemical potential of ROS is determined by the rates of production, interconversion, mobility, and ultimate annihilation of ROS, and is related to the time-averaged concentration of ROS in the vicinity, and over the lifetime, of any ROS-sensitive marker. One such marker, which both is sensitive to ROS (*e.g.*, $\text{O}_2^{\bullet-}$) and provides a distinctive EPR signal, is aconitase. Functional aconitase contains an EPR-silent $[\text{4Fe4S}]^{2+}$ cluster that contains a uniquely labile Fe_a atom that is bound to the cluster solely via inorganic sulfide. Upon reaction with $\text{O}_2^{\bullet-}$, Fe_a is lost and a catalytically inactive but EPR-active $[\text{3Fe4S}]^+$ cluster remains that exhibits a sharp and distinct, almost-isotropic signal centered at $g = 2.018$ (45,72–75). This signal overlaps with the oxidized S3 $[\text{3Fe4S}]^+$ signal from complex II, and care must be taken to resolve the signals by temperature dependence where measurements of the reduced complex I $[\text{2Fe2S}]^+$ and $[\text{4Fe4S}]^+$ signals indicate an elevated mitochondrial redox potential (44). However, the aconitase $[\text{3Fe4S}]^+$ signal is still a very convenient and

sensitive marker of ongoing oxidative stress, and its magnitude has been observed to increase by up to a factor of 10 over the basal level (21).

(iii) Chronic exposure to ROS may, in some cases, be accompanied by the appearance of signals flanking the $g = 6$ ferriheme signal that are due to ferriheme in catalase. Catalase expression is known to protect against oxidative stress, and catalase overexpression protects against ROS-mediated cellular damage (76–79). Because catalase is not constitutively expressed at EPR-detectable levels in most tissues (liver is an exception (44)), the appearance of catalase EPR signals over time, or in response to stress, provides an oxidative biomarker during sustained exposure to ROS. Free radical signals of high amplitude are also detected in some tissue samples, indicating the presence of stable free radicals at the time of freezing. These are usually accompanied by severely attenuated FeS signals, and studies elsewhere on irradiated biological membranes suggest that these signals are likely due to amino acid radicals in membrane proteins (80).

Detection of dinitrosyl iron complexes as a potential target of NO

Under conditions where NO is generated, low-temperature EPR can detect nitrosylated paramagnetic species. Low-temperature EPR is used to detect the dinitrosyl iron complex formed by nitrosation (or nitrosylation) of non-heme protein by NO or NO-derived species in acute cardiac allograft rejection (81). Typically, EPR signals at $g=2.04$ and 2.02 were observed (82). These signals were inhibited in the presence of inducible nitric oxide synthase (Inos) inhibitor, suggesting that NO or NO-derived species were responsible for the formation of dinitrosyl iron complex. NO and oxidants derived from it could also induce oxidation of iron sulfur clusters, including the 4Fe-4S cluster in aconitase (83,84). Low-temperature EPR spectroscopy can be used to assess NO formation (inhibitible by Inos inhibitors) in tumors subjected to increased vascular permeability and blood flow (85). In blood perfused models, EPR can detect an iron nitrosyl complex (NO-hemoglobin or HbNO) as a surrogate marker of Inos activity (85).

A proof of concept: EPR detection of biomarkers of mitochondrial redox changes in pancreatic cancer MiaPaCa-2

A recent study illustrates the utility of EPR at cryogenic temperatures for studying the role of ROS and oxidation in cancer biology research (21). Figure 1 shows the effect of incubation of pancreatic cancer MiaPaCa-2 cells with the mitochondria-targeted agent metformin (Mito-Met) (86). The left panel shows the experimental EPR spectra over the field envelope containing the reduced complex I $[2\text{Fe}_2\text{S}]^+$ and $[4\text{Fe}_4\text{S}]^+$ signals and the aconitase $[3\text{Fe}_4\text{S}]^+$ signal, and the right panel shows the spin concentration due to oxidatively damaged aconitase (bottom) and the total spin concentration due to reduced FeS clusters N1b, N2, N3, N4, S1, and S2 (top), extrapolated from the measurement of the $g = 1.92$ signal. Markers are shown for the resonant-field positions of aconitase $[3\text{Fe}_4\text{S}]^+$ (“Acn”), complex II S3 $[3\text{Fe}_4\text{S}]^+$, the $g = 1.92$ composite signal (“FeS”), and the complex I N4 $[4\text{Fe}_4\text{S}]^+$ $g(x)$ resonance (“N4b”). The measured (integrated) intensity of the $g = 1.92$ signal decreases approximately exponentially with increasing Mito-Met, indicating a Mito-Met-dependent increase in redox potential and consequent decrease in metabolic potential.

Closer inspection of the spectra indicates that the N4 $[4\text{Fe}4\text{S}]^+$ $g(x)$ resonance falls off far more rapidly with [Mito-Met], consistent with its midpoint potential, and that the $g = 1.92$ signal becomes less sharp with increasing [Mito-Met] as the lower potential complex I clusters become oxidized prior to the complex II S1 and S2 clusters. Additionally, an $m_I = -3/2$ resonance of the signal from the $m_S = 1/2$ manifold of $S = 5/2$ Mn^{2+} overlaps the $g = 1.92$ signal (the corresponding $m_I = -5/2$ resonance entirely prevents identification of a $g(x)$ resonance from N3), and the reported total spin concentration of the reduced FeS clusters most realistically represents an upper limit. Because the metabolic potential of the mitochondria in MiaPaCa-2 cells is diminished by incubation with increasing concentrations of Mito-Met, the extent of oxidative damage to the FeS cluster of aconitase is seen to correspondingly increase, reaching saturation at 3 μM Mito-Met. EPR then shows that (i) ROS are produced in MiaPaCa-2 cells in a dose-responsive manner upon incubation with Mito-Met, and (ii) loss of metabolic function occurs concomitantly, with a reasonable correlation to detected ROS.

Figure 2 shows the analogous EPR data from MiaPaCa-2 cells upon incubation with the classic complex III inhibitor antimycin A (87–89). As with Mito-Met, a progressive increase in the signal from the ROS-sensitive biomarker, aconitase $[3\text{Fe}4\text{S}]^+$, is observed with increasing concentrations of antimycin A. However, in marked contrast to Mito-Met, no effect on the mitochondrial complexes I and II redox potential is observed. Mito-Met and antimycin A act in very different ways: Mito-Met is a mitochondrially targeted agent and penetrates the mitochondrial membranes prior to inducing ROS production; EPR detects both a compromised mitochondrial redox potential and ROS-dependent oxidative stress. Antimycin A acts by binding to the ubiquinol-binding Q_i site of complex III and preventing ubiquinone turnover. This inhibition destroys the ability of the electron transport chain to function to maintain a proton gradient across the inner mitochondrial membrane, a consequence of which is the formation of superoxide in the absence of an electron acceptor, but neither stops the flow of electrons into complexes I and II nor physically compromises the mitochondrial membranes in a manner that leads to collapse of the mitochondrial redox potential. The ability of EPR to elegantly discriminate between the discrete methods of action of Mito-Met and antimycin A serves to illustrate its potential in cancer biology research and redox therapy evaluation. These results obtained under “probe-free” conditions were validated using exogenously added ROS probes (21).

We recently showed that molecules (honokiol, apocynin, metformin) present in naturally occurring plants can be targeted to mitochondria by modification of their chemical structures. The modification involves conjugating the untargeted molecule to a triphenylphosphonium moiety through an aliphatic linker chain (90). These compounds are known as mito-honokiol, mito-apocynin, or Mito-Met. Mito-Met inhibits the proliferation of pancreatic cancer cells at concentrations that are >100 fold lower than that of metformin and also inhibited tumor growth in mice xenograft model (86). Mito-honokiol inhibited the metastasis of lung cancer to the brain in a mouse xenograft model (91). The proposed mechanism involves mitochondrial ROS formation resulting from Mito-Met or mito-honokiol mediated inhibition of mitochondrial complex I (91). Whereas this mechanism can be demonstrated by direct low-temperature EPR and by using redox active fluorescent probes in tumor cells, at present *ex vivo* EPR is possibly the only available technique to

demonstrate oxidant formation in tumor tissues in response to drug therapy or drug resistance. Mitochondria-targeted drugs are more selectively sequestered in tumor cells as compared with normal cells and are relatively nontoxic. For example, mito-honokiol does not show toxicity when administered to mice in amounts 20 times higher than the effective dose (91).

OXPHOS-targeted antitumor drugs

At this time, low-temperature EPR is probably the only probe-free technique that is capable of assessing metabolic reprogramming and the effect of metabolic intervention in frozen intact tumor tissues. Cancer cells support their increased energetic needs by altering their metabolism. Typically, cancer cells use glycolytic pathways to acquire energy (the Warburg effect) for sustaining proliferation and growth. However, in response to therapeutic inhibition of BRAF(V600E) mutations, melanoma cells reverse metabolic reprogramming, resulting in elevated OXPHOS (4). Metabolic reprogramming also leads to resistance to the drug, vemurafenib, in melanoma cells. It was proposed that well-tolerated inhibitors of OXPHOS are potentially useful therapeutic modulators (*e.g.*, metformin, phenformin) (92).

Conclusions

In this mini review, we have discussed the significance of ROS formation in drug-resistant tumors and in immunotherapy. As recently discussed in a methods paper on mitochondrial ROS formation (21), the *ex vivo* low-temperature EPR technique is an ideally suitable technique for monitoring oxidant formation and redox changes in tumor mitochondria. This probe-free EPR methodology can easily be extended to measure oxidant formation in human tumors.

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Figure 1.

Low-temperature EPR of the oxidized aconitase and reduced iron sulfur centers of mitochondrial complexes I and II induced by Mito-Met in MiaPaCa-2 cells. Cells were treated with Mito-Met for 24 h, collected, and stored at -80°C until EPR measurements. The signal labeled “Acn” is assigned to the oxidatively modified $[3\text{Fe-4S}]^{+}$ cluster from aconitase. The signal labeled “FeS” is assigned to the reduced $[2\text{Fe-2S}]^{+}$ and $[4\text{Fe-4S}]^{+}$ clusters from complexes I and II. The graphs show the quantitative changes in EPS signal intensity due to FeS and Acn. T-test vs. control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (This research was originally published in the Journal of Biological Chemistry. G. Cheng, M. Zielonka, B. Dranka, S.N. Kumar, CR. Myers, B. Bennett, A.M. Garces, L.G. Dias Duarte Machado, D. Thiebaut, O. Ouari, M. Hardy, J. Zielonka, B. Kalyanaraman. Detection of mitochondria-generated reactive oxygen species in cells using multiple probes and methods: Potentials, pitfalls, and the future. J. Biol. Chem. 2018; [in press]. © the American Society for Biochemistry and Molecular Biology.)

Figure 2.

Low-temperature EPR of the oxidized aconitase and reduced iron sulfur centers of mitochondrial complex 1/2 induced by antimycin A in MiaPaCa-2 cells. Cells were treated with antimycin A for 24 h and stored at -80°C prior to EPR measurements. Experimental conditions and description are the same as in Figure 1 (This research was originally published in the Journal of Biological Chemistry. G. Cheng, M. Zielonka, B. Dranka, S.N. Kumar, C.R. Myers, B. Bennett, A.M. Garces, L.G. Dias Duarte Machado, D. Thiebaut, O. Ouari, M. Hardy, J. Zielonka, B. Kalyanaraman. Detection of mitochondria-generated reactive oxygen species in cells using multiple probes and methods: Potentials, pitfalls, and the future. J. Biol. Chem. 2018; [in press]. © the American Society for Biochemistry and Molecular Biology.)