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ORIGINAL PAPER

An EPR Study Using Cyclic Hydroxylamines To Assess The Level of Mitochondrial ROS in Superinvasive Cancer Cells

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Abstract

It has been proposed that a mitochondrial switch involving a high mitochondrial superoxide production is associated with cancer metastasis. We here report an EPR analysis of ROS production using cyclic hydroxylamines in superinvasive SiHa-F3 compared with less invasive SiHa wild-type human cervix cancer cells. Using the CMH probe, no significant difference was observed in the overall level of ROS between SiHa and SiHa-F3 cells. However, using mitochondria-targeted cyclic hydroxylamine probe mitoTEMPO-H, we detected a significantly higher mitochondrial ROS content in SiHa-F3 compared with the wild-type SiHa cells. To investigate the nature of mitochondrial ROS, we overexpressed superoxide dismutase 2, a SOD isoform exclusively localized in mitochondria, in SiHa-F3 superinvasive cells. A significantly lower signal was detected in SiHa-F3 cells overexpressing SOD2 compared with SiHa-F3. Despite some limitations discussed in the paper, our EPR results suggest that mitochondrial ROS (at least partly superoxide) are produced to a larger extent in superinvasive cancer cells compared with less invasive wild-type cancer cells.

Introduction

A few years ago, it has been proposed that a mitochondrial switch involving a high mitochondrial superoxide production was associated with cancer metastasis [1]. In their study, Porporato et al. developed a superinvasive human cervix carcinoma cell line (called SiHa-F3) through several rounds of in vitro selection in transwells. Compared with wild-type SiHa cells, this variant cell line presented an enhanced invasive activity and an aberrant tricarboxylic acid cycle activity. Notably, increased cell respiration in the superinvasive variant was proposed to produce increased amounts of mitochondrial superoxide that triggered the prometastatic tumor-transforming growth factor β pathway via activation of src kinase directly in mitochondria [1]. Accordingly, pharmacological approaches creating a bottleneck in the electron transport chain (ETC) increased mitochondrial superoxide production and also promoted cancer cell migration, invasion, and metastasis. Conversely, the migration activity of SiHa-F3 cells and pharmacologically treated cells were inhibited when treated with mitochondrial superoxide scavenger mitoQ [1].

Of note, in this paper [1], the evidence for reactive oxygen species (ROS) production was based on fluorescence measurements using mitoSOX. MitoSOX is a widely used probe that contains a dihydroethidium structure linked to a triphenylphosphonium moiety that allows its accumulation in mitochondria. Despite its high sensitivity for mitochondrial superoxide detection, some drawbacks restrict its use because mitoSOX can form two fluorescent products with overlapping spectra, with only one of them being superoxide specific [2–6]. Consequently, the claim for a higher superoxide production in the cancer cells on the basis of simple fluorescence assay using mitoSOX has been recently challenged [7].

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Besides fluorimetry, electron paramagnetic resonance (EPR) could potentially provide information on ROS and superoxide production. Recently, a study compared the sensitivity of different EPR methods to detect superoxide in buffer, cell lysates and cells [8]. It was found that spin traps and cyclic hydroxylamines (pending the use of appropriate SOD controls) were good candidates to sensitively detect superoxide in complex biological media, the cyclic hydroxylamines offering the best sensitivity.

As measuring the level of superoxide produced in the mitochondria of cancer cells is a matter of debate, we sought to assess mitochondrial ROS production using EPR spectroscopy. During the XIth International Workshop on EPR in Biology and Medicine (Krakow, October 2019), we reported an EPR analysis of ROS production in superinvasive SiHa-F3 cells compared with less invasive SiHa wild-type cancer cells. On the one hand, overall ROS production was analyzed using CMH, a probe that distributes in the extra- and intracellular compartments (Fig. 1), associated with PEG-SOD in control experiments. On the other hand, mitochondrial ROS production was assessed using two cyclic hydroxylamines coupled to a triphenylphosphonium moiety providing an accumulation in the mitochondrial compartment: mitoTEMPO-H [9] and mitoCPH [10] (Fig. 1). In addition, to get insight on the nature of mitochondrial ROS detected in superinvasive cells, we overexpressed the superoxide dismutase 2 (SOD2), a manganese-dependent SOD isoform exclusively localized in mitochondria. Overall, our EPR results suggest that mitochondrial ROS (at least partly superoxide) are produced to a larger extent in superinvasive cancer cells compared with less invasive wild-type cells.

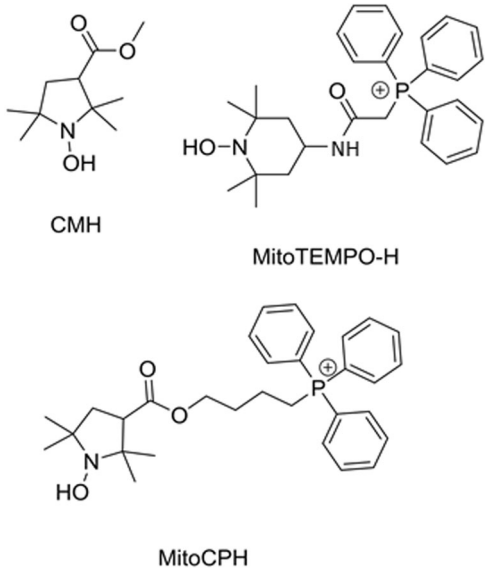

Fig. 1 Chemical structures of the EPR probes used in the present study

Material and Methods

Cells and Reagents

SiHa human cervix adenocarcinoma cells were from the American Type Culture Collection (ATCC, Manassas, VA, USA). Superinvasive SiHa-F3 cells were obtained following 3 rounds of in vitro selection starting from SiHa cells, as previously described [1]. CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) and mitoTEMPO-H (1-hydroxy-4-[2-(triphenylphosphonio)-acetamido]-2,2,6,6 tetramethylpiperidine) were from Enzo Life Sciences (Antwerpen, Belgium); SOD conjugated with polyethylene glycol (PEG-SOD) and diethylene triamine pentaacetic acid (DTPA) from Sigma Aldrich (Overijse, Belgium); and PBS from ThermoFisher (Merelbeke, Belgium, catalog #10010023). MitoCPH (3-(((carbonyl)oxy)butyl-triphenylphosphonium)-2,2,5,5-tetramethylpyrolidinooxy bromide) was synthesized as previously described [8].

Measurement of Mitochondrial ROS Production

Subconfluent cells were harvested and resuspended in Krebs HEPES Buffer (pH 7.4) at 20×10^6 cells/mL. The experimental mixture was prepared with cells, DTPA 1 mM, and the probe (MitoCPH or MitoTEMPO-H, 160 µM). The sample was then transferred into a gas-permeable polytetrafluoroethylene (PTFE) tubing that was inserted in a quartz tube with both extremities open. Of note, this process took 1–2 min before the tube was placed in the cavity of the EPR system previously equilibrated at 310 K. Increase in signal amplitude was monitored during 10 min after the addition of the probe. Calibration curves were obtained by plotting the amplitudes of the low-field component of EPR spectrum against various nitroxide concentrations. Data are expressed as the difference between nitroxide concentration obtained at 10 min and the initial nitroxide concentration, as previously described [10].

Measurement of Cytoplasmic Superoxide Production

Subconfluent cells were harvested and resuspended in Krebs HEPES Buffer (pH 7.4) at 20×10^6 cells/mL. The experimental mixture was prepared with cells, DTPA 1 mM and CMH 0.5 mM. PEG-SOD 200 U/mL (5 min preincubation before addition of cyclic hydroxylamines) was added in control experiments. EPR signal intensity was monitored during 10 min.

Overexpression of SOD2

SiHa-F3 cells were transfected by lentiviruses containing a SOD2 gene with a CMV promotor. The lentiviruses were

generated using the vector psi-LVE001 (reference: LPP-HSE110104-LVE001-050; GeneCopoeia), according to manufacturer's instructions. Multiplicity of infection was 40 and the infection time was 16 h. A western blot was performed to confirm the overexpression of SOD2 in the cells, following a previously disclosed protocol [11]. Briefly, whole-protein extracts were prepared using RIPA buffer containing phosphatase and protease inhibitors (Sigma Aldrich) and quantified using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). Protein lysates (12 µg) were loaded in a 15% polyacrylamide gel. After migration, protein transfer was performed on PVDF membranes. Used primary antibodies included rabbit polyclonal against SOD2 (catalog #ab13533, Abcam, Cambridge UK) and a mouse monoclonal against β-actin (catalog #A5441, Sigma Aldrich). Secondary antibodies against rabbit and mouse were from Sigma Aldrich. Detection was performed with the ECL system (Amersham, UK).

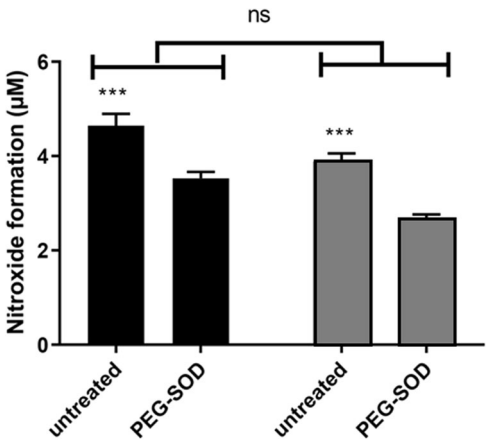

Fig. 2 Overall ROS production estimated with CMH in SiHa to SiHa-F3 cancer cells. SiHa and SiHa-F3 cells were treated (or not) for 10 min using 200 U/mL of PEG-SOD. The graphs show the nitroxide concentration detected after 10 min in SiHa (black) and SiHa-F3 (light gray) using 0.5 mM of CMH ($n = 3$). ***$P < 0.001$, ns > 0.05 by two-way ANOVA with Turkey post-hoc test

EPR Experiments

EPR measurements were performed using a Bruker EMX-Plus spectrometer (Bruker, Rheinstetten, Germany) operating in X-band (9.85 GHz) and equipped with a PremiumX ultra low noise microwave bridge and a SHQ high sensitivity resonator. The tube containing the PTFE tubing was directly inserted in the EPR cavity, which was heated at 310 K with air during all experiments. All experiments were done in triplicate. Typical settings were as follows: microwave power: 20 mW; modulation frequency: 100 kHz; modulation amplitude: 0.1 mT; time constant: 20.48 ms; conversion time: 20.00 ms; data points: 1380; sweep width: 5.3 mT.

Statistics

All data are shown as means ± SEM. *n* refers to the total number of replicates per group. One-way ANOVA and two-way ANOVA were used to determine statistical differences. $P < 0.05$ was considered to be statistically significant.

Results

Overall ROS Production in Poorly Invasive and Superinvasive Cancer Cells

The overall ROS production by SiHa and SiHa-F3 cells was determined using the oxidation of CMH into nitroxide [9, 12]. CMH is well known to be distributed in the intra- and extracellular spaces [9]. To assess the contribution of superoxide to nitroxide formation from the hydroxylamine, PEG-SOD was used as a control. Figure 2 shows the nitroxide concentration obtained after 10 min of incubation in the absence or in the presence of PEG-SOD. No significant difference was observed in the overall level of ROS between SiHa and SiHa-F3 cells. Of note, there was a significant decrease in the formation of nitroxide in the presence of PEG-SOD (Fig. 2).

Mitochondrial ROS Detection in Poorly Invasive and Superinvasive Cancer Cells

We compared the basal level of mitochondrial ROS in SiHa and SiHa-F3 cell using mitochondria-targeted cyclic hydroxylamine probes mitoCPH and mitoTEMPO-H in EPR measurements. Using mitoCPH, no significant difference was observed between the two cell lines (Fig. 3b). However, mitoTEMPO-H allowed the detection of a significantly higher mitochondrial ROS content in SiHa-F3 compared with the wild-type SiHa cells (Fig. 3c).

Nature of the Mitochondrial ROS Detected in Superinvasive Cancer Cells

To investigate the nature of mitochondrial ROS detected in the previous experiment, we overexpressed SOD2 in the SiHa-F3 superinvasive cells. Overexpression was confirmed using western blotting (Fig. 3a). When mitoCPH was tested, no significant differences were observed between both cell lines (Fig. 3b). However, using mitoTEMPO-H, a significant lower signal was detected in SiHa-F3 cells overexpressing SOD2 compared with SiHa-F3 (Fig. 3c).

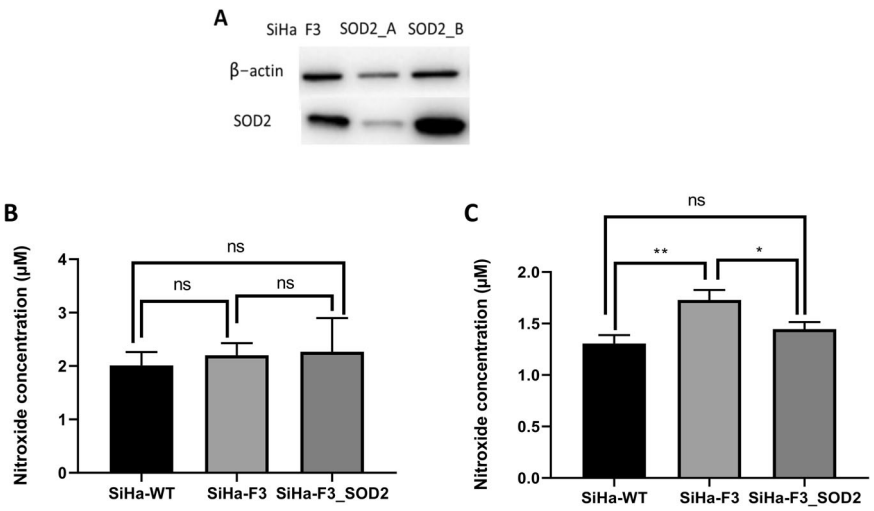

Fig. 3 Mitochondrial ROS production estimated with mitoCPH and mitoTEMPO-H in cancer cells. SiHa-F3 cells transduced, or not, with a lentiviral vector that constitutively induced SOD2 overexpression (SiHa-F3_SOD2) were compared with wild-type SiHa cells. **a** Representative western blot showing SOD2 and β-actin expression in SiHa-F3 and SiHa-F3_SOD2 cells. SOD2_B was selected to perform the EPR experiments. **b** Nitroxide concentration reached after 10 min using mitoCPH 160 μM ($n = 3$). **c** Same as in **b**, but using mitoTEMPO-H 160 μM ($n = 3$). ns $P > 0.05$, $^*P < 0.05$, by one-way ANOVA

Discussion

Mitochondrial superoxide has been suggested to promote cancer metastasis [1, 13, 14]. In order to document its possible involvement in the metastatic process, we used the cancer cells previously studied by Porporato et al. [1], namely poorly invasive SiHa and superinvasive SiHa-F3 cells. In the present study, we assessed ROS production using the conversion of cyclic hydroxylamines to nitroxides.

Using CMH, a probe that distributes in the intra- and extracellular spaces, we first observed no significant difference in global ROS levels between SiHa and SiHa-F3 cells (Fig. 2). It is worth to be noticed that the formation of nitroxides could involve additional mechanisms to superoxide detection, including unspecific oxidation mechanisms or reaction with other ROS species. Hydroxylamines can indeed be oxidized by superoxide, but also H_2O_2 in the presence of HRP or metal ions, peroxynitrite, and derived nitrogen species [8, 15, 16]. It was also described that lipophilic hydroxylamines are significantly oxidized by enzymes in the mitochondrial ETC, mainly by cytochrome c oxidase [17]. Therefore, a control experiment was performed with PEG-SOD to ascribe superoxide contribution to nitroxide formation. As we observed a significant decrease in nitroxide formation levels in the presence of PEG-SOD, this suggests that superoxide contributes, in part, to the ROS level measured.

We then focused on mitochondrial ROS production using two cyclic hydroxylamines that accumulate in mitochondria. Using mitochondria-targeted cyclic hydroxylamine probe mitoCPH, we did not observe any significant difference in nitroxide formation between SiHa and SiHa-F3 cells (Fig. 3b). In contrast, using mitoTEMPO-H, we observed a significantly higher mitochondrial ROS production in SiHa-F3 compared with SiHa cells (Fig. 3c). To further analyze the nature of these ROS, we overexpressed SOD2 in SiHa-F3 cells. We observed a decrease in ROS production in SiHa-F3_SOD2 cells compared with SiHa-F3 cells (Fig. 3c). As SOD2 is specifically scavenging superoxide, these results suggest that ROS produced in the mitochondrial compartment are, at least partly, superoxide radicals.

Several factors could explain the difference of behavior between mitoCPH and mitoTEMPO-H. First, while mitoCPH was previously found to be more sensitive than mitoTEMPO-H in the presence of high superoxide fluxes (using a hypoxanthine/xanthine oxidase system producing around 2 μM superoxide/min in PBS), both probes presented similar performance at low superoxide fluxes (e.g., 0.1 μM superoxide/min) [8, 10]. Second, mitoTEMPO-H may have a higher reactivity with superoxide compared with mitoCPH since piperidine-derived hydroxylamines have higher constant rates for reactions with superoxide compared with pyrrolidine-derivatives [9, 18]. Therefore, higher reactivity of mitoTEMPO-H can potentially explain the better detection of superoxide in the cancer cell mitochondria. Third, it is possible that the ester link of mitoCPH could be hydrolyzed in cells, leading to a lower accumulation of the probe in mitochondria compared with mitoTEMPO-H and, consequently, to a lower performance for ROS detection despite a higher resistance to reduction of pyrrolidine nitroxides compared with piperidine nitroxides [19]. Fourth, it is likely that mitoCPH and mitoTEMPO-H

are not localized in the same position within the mitochondrial matrix. Indeed, the analysis of the EPR spectra of mitoCP° [10] and mitoTEMPO° [18] in isolated mitochondria reveals different degree of immobilization for mitoCPH and for mitoTEMPO-H. Due to its longer alkyl chain, mitoCPH could remain attached to the inner mitochondrial membrane whereas mitoTEMPO-H is not.

A possible limitation of the present study relies on the high concentrations of probes (160 µM) that were used, calling for caution about data interpretation. The concentration of the probes is likely even higher in the mitochondrial compartment due to its accumulation thanks to the triphenylphosphonium moiety. Previous studies showed that a similar structure, mitoCP, could interfere with mitochondrial bioenergetics at concentration as low as 1 µM [20]. In addition, it was suggested that the linker length may play a role in mitochondrial activity [21]. Therefore, we cannot exclude that our results may be contaminated by artificial ROS production caused by an interaction of the probe with mitochondrial enzymes or by a potential disruption in mitochondrial function. Also, the difference in the mitochondrial bioenergetics between SiHa and SiHa-F3 may also account for the differences observed in both cell lines. In addition, the effects of SOD2 overexpression on cellular bioenergetics have not yet been investigated. For that purpose, further experiments will be necessary to evaluate mitochondrial integrity and bioenergetics. Finally, we should keep in mind that the concentration of the nitroxide results from the competition between oxidation of the hydroxylamine and the reduction of the nitroxide by reductants present in the cellular systems. Our interpretation of the results supposes that the reduction potency does not vary between the cellular systems, an assumption that will require further analysis.

In conclusion, the two main results of the present study are that (1) among cyclic hydroxylamines, mitoTEMPO-H has a better performance than mitoCPH to detect mitochondrial ROS in cells, and that (2) mitochondrial ROS (at least partly, superoxide) are likely to be produced to a larger extent in superinvasive human cervix SiHa-F3 cancer cells compared with poorly invasive SiHa cells. This higher ROS production seems confined to the mitochondrial compartment. Our results also suggest that superoxide is produced as the overexpression of mitochondrial superoxide dismutase SOD2 inhibited the EPR signal coming from the oxidation of mitoTEMPO-H. This later result is in agreement with Porporato et al. [1] who previously proposed the involvement of mitochondrial ROS in metastasis. Further studies, using mitoSOX coupled with HPLC, could potentially confirm our results.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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