Techniques for Detecting Reactive Oxygen Species in Pulmonary Vasculature Redox Signaling

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1 Redox Signaling and Reactive Oxygen Species in Pulmonary Vasculature

A large body of evidence suggested that redox signaling play important roles in regulating pulmonary vasculature function, during which various reactive oxygen species (ROS) are involved [1–15]. As important cellular signaling molecules, the ROS, e.g., superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), lipid peroxyl radical ($LOO^{\bullet}$), and peroxynitrite ($ONOO^-$), could be generated from a variety of sources in cells including NADPH oxidase, uncoupled endothelial nitric oxide synthase, xanthine oxidase, cyclooxygenase, lipoxygenase, cytochrome P450, and the mitochondrial respiratory chain [1, 2, 11–15].

Aberrant redox signaling, e.g., overproduction of reactive oxygen species that exceeds the capability of cellular antioxidant mechanisms, has been found to alter normal vasculature function and remodel blood vessel structure, thus contributes to pathological processes of pulmonary vasculature [1–15]. In fact, increase of ROS and related enzyme expression levels have been commonly found in pulmonary arterial hypoxia and hypertension models, and the mechanism by which ROS influence on pulmonary vasculature function could vary in many aspects [1–15]. For example, evidence showed that hypoxic exposure led to an increase of mitochondria-derived ROS, which further triggered a rise of intracellular Ca^{2+} level and caused pulmonary vasoconstriction, thereby resulting in an increase in pulmonary vascular resistance [3–6]; production of ROS such as $O_2^{\bullet -}$ and H_2O_2 were found to contribute to blood vessel remodeling by affecting pulmonary smooth muscular cell proliferation and apoptosis [7, 8]; the excessive production of $O_2^{\bullet -}$ also led to a decrease of levels of nitric oxide and prostacyclin, two major vasodilating factors, which consequently induced endothelial dysfunction [9, 10]. Therefore, the regulation of pulmonary vasculature via redox signaling, especially through the production of ROS, is a very complicated process with various biological events involved, and technical advance in the study of ROS will result in a better understanding on how to maintain normal pulmonary vasculature functions under oxidative stress.

Although substantial evidence suggested that redox signaling is tightly implicated in the physiological and pathological processes of pulmonary vasculature, the specific effect of individual ROS and the underlying mechanism

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Y.-X. Wang (ed.), *Pulmonary Vasculature Redox Signaling in Health and Disease*,
Advances in Experimental Medicine and Biology 967, DOI 10.1007/978-3-319-63245-2_23

still remain unclear. This is mainly because of the extremely short lifetime of ROS, which makes it very difficult to monitor the free radicals and investigate their bioactivity. Therefore, developing specific and sensitive methods to detect ROS in complex biological system is essential for us to advance our knowledge in pulmonary vasculature regulation.

Here we introduced and summarized commonly used techniques for the detection of ROS in vitro and in vivo, including chemiluminescence-based assay, fluorescence-based assay, cytochrome c reduction, genetically encoded fluorescent probes, as well as ESR/spin trapping technique which has great potential and requires additional attention in future ROS study. Their individual advantages and limitations as well as recent technical advance were also discussed.

2 Techniques for Detecting Reactive Oxygen Species in Pulmonary Vasculature

2.1 Chemiluminescence Assay

Chemiluminescence-based assay is a commonly used method for the detection of cellular production of ROS. During a chemiluminescence assay, the ROS in cell or tissue samples are allowed to react with certain chemiluminescent probes, such as lucigenin, luminol, and diogenes, to produce oxidative end products, which could lead to emission of light that can be measured and quantified by a luminometer [16–25]. For instance, during lucigenin-enhanced chemiluminescence assay, ROS such as $O_2^{\bullet-}$ could firstly reduce lucigenin (LUC^{2+}) to lucigenin cation radical ($LUC^{\bullet+}$), then a second $O_2^{\bullet-}$ turns $LUC^{\bullet+}$ to lucigenin dioxetane ($LUCO_2$) which further breakdown to N-methylacridinium followed by emission of a photon (Scheme 1) [16, 24, 25]. The production of light could thus be monitored and quantified as a measurement of the $O_2^{\bullet-}$ level in the system.

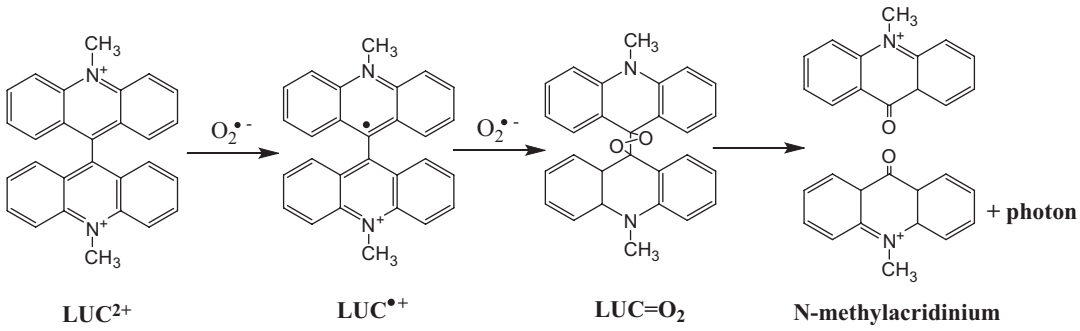
Among all of the chemiluminescent probes, lucigenin is the most commonly used probe for ROS detection in pulmonary vasculature study due to its specificity to superoxide. For example, to investigate the contribution of ROS in altered pulmonary vascular responses in piglets with pulmonary hypertension, Fike et al. employed lucigenin-enhanced chemiluminescence to detect and quantify production of ROS in dissected piglet pulmonary arteries with chronic hypoxia-induced hypertension [18]; Liu et al. used lucigenin-enhanced chemiluminescence to measure $O_2^{\bullet-}$ levels in isolated mouse intrapulmonary arteries [19]; Tickner et al. also used lucigenin chemiluminescence to measure the $O_2^{\bullet-}$ production in homogenized mouse lung tissue, in which inhibitors for various $O_2^{\bullet-}$-producing enzymes was employed to identify the enzymatic sources of $O_2^{\bullet-}$ [20].

Although it has been widely applied in various studies for ROS detection, lucigenin-enhanced chemiluminescence also suffers from a critical problem. During the reaction between lucigenin and $O_2^{\bullet-}$, the intermediate lucigenin radical $LUC^{\bullet+}$ could also react with oxygen molecule to produce additional $O_2^{\bullet-}$ [16, 17]. This side reaction leads to artificial overestimation of the superoxide in the system, making the results of this assay problematic.

In addition to lucigenin, there are also other chemiluminescent probes such as luminol, diogenes, and cypridina luciferin analog, which can react with a variety of ROS, including $O_2^{\bullet-}$, H_2O_2, hydroxyl radical and peroxynitrite [16, 17, 21–23]. By comparing their various reaction specificities, one may choose suitable probes for different detection purposes. In addition, instead of using a single probe in the experiment, Yamazaki et al. showed that combination of two chemiluminescent probes, e.g., luminol and diogenes, could work together synergistically to detect ROS in suspended neutrophils with a higher sensitivity [22].

2.2 Fluorescence-Based Methods for ROS Detection

During a fluorescence-based assay for ROS detection, the cell or tissue samples are incubated with various fluorescence probes to produce fluorescent end products, which could be detected and quantified by fluorescent microscopy or plate



Scheme 1 Reaction between lucigenin and $O_2^{\bullet-}$ during lucigenin-enhanced chemiluminescence assay

reader. There are many types of fluorescence probes with specificities towards different types of ROS as well as particular subcellular locations. For example, dihydroethidium (DHE) is commonly used for the detection of cellular production of $O_2^{\bullet-}$ because it can react with $O_2^{\bullet-}$ to form a specific fluorescent adduct 2-hydroxyethidium, which can be detected at 605 nm when excited at 518 nm [7, 17, 26, 27]. Dichlorodihydrofluorescein diacetate (DCFH-DA) is a commonly used fluorescence probe for detecting intracellular ROS due to its ability to rapidly diffuse across cell membranes. Once uptaken into cells, DCFH-DA can be hydrolyzed by intracellular esterase to form dichlorodihydrofluorescein (DCFH), which is retained inside of cells and oxidized by the intracellular ROS, e.g., H_2O_2, $O_2^{\bullet-}$, $ONOO^-$, and lipid hydroperoxides, to produce a fluorescent product dichlorofluorescein DCF [7, 17, 20, 28, 29]. Dihydrorhodamine (DHR) is another commonly used fluorescence probe for detection of ROS, especially peroxide and $ONOO^-$. It can be oxidized by ROS to produce a two-electron oxidized fluorescent product, rhodamine, which can be monitored by fluorescence spectroscopy with excitation and emission wavelengths of 500 and 536 nm, respectively [17, 30, 31]. Another commonly used fluorescence-based method is Amplex Red assay, which is a specific and sensitive method for detecting extracellular ROS, especially H_2O_2. During the assay, Amplex Red (*N*-acetyl-3, 7-dihydroxyphenoxazine) will be oxidized by H_2O_2 to form resorufin at the presence of horseradish peroxidase. When excited at 530 nm, the fluorescence of resorufin could be detected at 590 nm as a measurement of H_2O_2 level [17, 32–34].

By choosing proper type of fluorescence probes, researchers can selectively detect the production of various ROS in cells. In fact, fluorescence probes have been widely used to detect ROS production in dissected lungs in animal studies [7, 20, 29]. For example, Nijmeh et al. employed DHE and DCFH-DA method to detect and quantify the ROS level in mouse lung with ischemia induced by pulmonary artery ligation, and results showed that left pulmonary artery ligation led to transient ROS upregulation which promoted ischemia-induced angiogenesis [29]; Wedgwood et al. employed DHE and DCFH-DA to quantify ROS level in fetal pulmonary arterial smooth muscle cells (FPASMCs) in order to investigate the contribution of ROS in the proliferation of FPASMCs [7]; Tickner et al. measured ROS generation in the mouse lung tissues in situ via DHE fluorescence in order to investigate the effect of Nox2-derived ROS in PPARγ signaling and cell-cycle progression [20]. All these studies suggested that fluorescence-based assay is a powerful tool for ROS detection in the study of pulmonary vasculature.

However, there are also many limitations for fluorescence-based ROS detection. During DHE assay, DHE can undergo nonspecific redox reactions and produce another fluorescent product ethidium, whose fluorescent spectra overlap with that of 2-hydroxyethidium, therefore interfering the experimental outcome [17, 26, 35]. In the DCFH-DA method, the redox reaction of DCFH and DCF can form a DCF radical, which could

then react with oxygen to produce $O_2^{\bullet-}$, thereby leading to an overestimation of ROS level. In addition, oxidation of DCFH by ROS is not a direct reaction, it requires peroxidase activity, transition metals, and heme enzymes, hence changes in these cellular conditions could affect the experimental outcome independent of ROS levels [16, 17, 36]. In DHR assay, the oxidation of DHR by $ONOO^-$ is mediated by an intermediate DHR radical, however, the DHR radical can be reduced by reductant in cellular system, thereby leading to an artificial underestimate of the $ONOO^-$ production [17, 31]. These limitations must be taken into consideration during experiment design and data interpretation when fluorescence-based assay is used for ROS detection.

In order to circumvent abovementioned drawbacks, many efforts have been made to improve fluorescence-based ROS detection. For instance, to avoid the interference from nonspecific redox reaction in DHE-based method, Dikalov et al. and other groups employed HPLC analysis to separate the specific fluorescent adduct 2-hydroxyethidium from the nonspecific byproduct ethidium [16, 17, 26, 35]. This adapted DHE/HPLC method thus became a specific method for the detection of cellular $O_2^{\bullet-}$ level. In addition, DHE was also chemically modified to offer specificity to detect ROS in specific subcellular location. For example, mitoSOX, an analog of DHE, was developed with preference to accumulate in mitochondria, thus became a relatively specific probe to selectively detect mitochondria superoxide [16, 17, 35, 37].

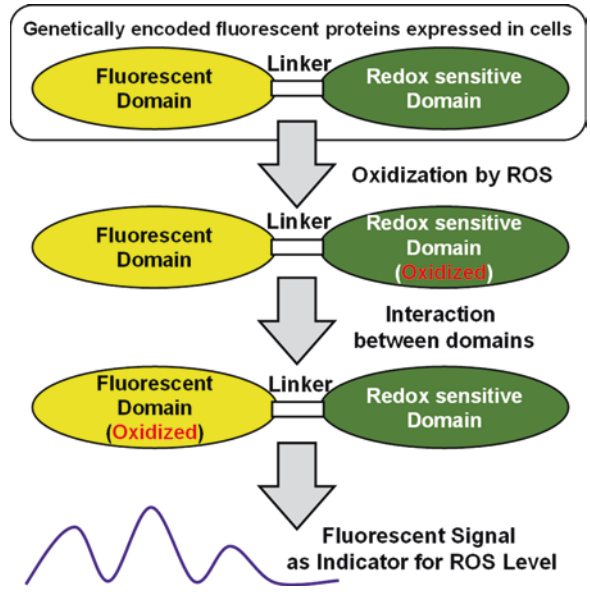
2.3 Cytochrome c Reduction

Cytochrome c reduction assay represents a classic method for detection of extracellular $O_2^{\bullet-}$ [16, 17, 38–44]. During the assay, cytochrome c-Fe^{3+} is reduced to cytochrome c-Fe^{2+} by receiving an electron from $O_2^{\bullet-}$, which can be observed by monitoring the changes in its spectrophotometric absorbance at 550 nm. At the meantime, the absorption at neighboring wavelength 540 and 560 nm remains same which could serve as isosbestic points. However, cytochrome c-Fe^{3+} can also be reduced by electrons donated from other enzymes and molecules in the cellular environment, which could interfere with the experiment outcome. To overcome this problem, the experiment must be performed in the presence and absence of superoxide dismutase (SOD), and the amount of $O_2^{\bullet-}$ should thus be calculated by comparing their difference, e.g., the SOD-inhibitable signals. The SOD/cytochrome c reduction method represents one of the most reliable method for specific detection of extracellular $O_2^{\bullet-}$ release, which has been applied in the study of pulmonary vasculature. For example, to investigate the contribution of bicarbonate-dependent superoxide release in pulmonary artery tone, Nozik-Grayck et al. used the SOD-inhibitable reduction of cytochrome c method to detect the extracellular $O_2^{\bullet-}$ in isolated rat pulmonary artery segments [44].

Although cytochrome c reduction is a well-recognized method for ROS detection, its relatively poor sensitivity may limit its application in the study of pulmonary vessels where the production of ROS is very low [16]. In addition, the presence of NO, $ONOO^-$, and peroxynitrous acid (ONOOH) in the system could oxidize cytochrome c-Fe^{2+} to cytochrome c-Fe^{3+}, thereby leading to underestimation of $O_2^{\bullet-}$ production [45].

2.4 Genetically Encoded Fluorescent Protein as Redox Indicators

In recent studies, genetically encoded fluorescent probes have been developed in which a fluorescent protein was linked to a ROS sensing protein to detect ROS inside living cells (Scheme 2) [17, 46–50]. For instance, Belousov et al. genetically inserted circularly permuted yellow fluorescent protein (cpYFP) into the regulatory domain of OxyR, a prokaryotic H_2O_2-sensing protein, to create a sensitive genetically encoded fluorescent probe, e.g., HyPer. The Hyper was found to be able to detect H_2O_2 production at the single cell level in the cytoplasm and mitochondria of HeLa cells [46]. Later on, Amit



Scheme 2 General principle for genetically encoded fluorescent protein as redox indicators [46–49]. The proteins containing a fluorescence domain and a redox sensitive domain are genetically encoded and expressed in cells. When the redox sensitive domain is oxidized by ROS, the fluorescence domain will undergo structural change or be oxidized by redox sensitive domain, thereby generating fluorescence signal

S et al. employed pHyPer-dMito (a modified version of HyPer which could exclusively target to mitochondrial inner membrane) to detect mitochondrial intermembrane space ROS generation in isolated pulmonary arterial smooth muscle (PASMCs) [47]. Gregory et al. linked enhanced cyan (CFP) and yellow (YFP) fluorescent protein motifs to the redox-dependent regulatory domain of bacterial heat shock protein HSP-33 to create a redox-sensitive fluorescence resonance energy transfer probe. After expressed in PASMCs, the oxidation of the thiols by ROS in HSP-33 domain led to a structural change in the optical coupling of CFP and YFP and a change in fluorescence signal, thus resulting in a sensitive, real-time assessment of changes in redox conditions in the cytosol [48]. Gutscher et al. incorporated a redox-sensitive green fluorescent protein roGFP2 into Orp1 protein, a seleno-independent member of the glutathione peroxidase family, to form orp1-roGFP2 fusion protein. The fusion protein could thus serve as a probe for H_2O_2 detection because H_2O_2-oxidized Orp1 could accept an electron from cysteine residues on the roGFP2, which then emits fluorescence [49]. Gregory et al. also directly expressed roGFP2 in different compartments of PASMCs, including cytosol, mitochondrial matrix and mitochondrial intermembrane space, in order to assess oxidant signaling in distinct intracellular compartments [50]. The genetically encoded fluorescent proteins have been demonstrated to result in specific, sensitive, and real-time measurement of intracellular ROS under physiologically relevant conditions, thus becoming a powerful tool in the study of pulmonary vasculature redox signaling.

2.5 Electron Spin Resonance

Electron spin resonance (ESR), or electron paramagnetic resonance (EPR), is a technique that detects the signals responding to the transition of unpaired electrons between different energy levels. When provided with certain value of energy from a magnetic cavity, the unpaired electrons in a compound of interest, e.g., a free radical species, will move between two energy states, and the ESR could monitor the absorption of energy and convert it into a spectrum [51, 52]. This characteristic makes ESR analysis a unique and conventional technique for the detection of free radical species.

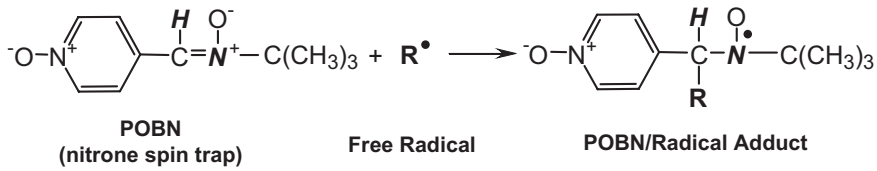
However, due to the high reactivity and short lifetimes in biological systems, the free radical species are usually undetectable by direct ESR analysis with only a few exceptions, such as ascorbyl,

tocopheroxyl and semiquinone radicals [51–53]. To overcome this shortcoming, spin probes, e.g., cyclic hydroxylamines, have been developed and applied to assist the ESR detection of free radical species [16, 17, 54–57]. These spin probes can be oxidized by free radical species to become ESR-active form with longer lifetime. For example, l-hydroxy-3-carboxy-2, 2, 5, 5-tetramethylpyrrolidine (CPH), a commonly used cyclic hydroxylamine spin probe, can be oxidized by superoxide to become a radical form $CP^{\bullet}$ and give ESR active signal [56]. The cyclic hydroxylamines spin probes can react with ROS with high rate constants, thus can be applied at low concentrations and result in high detection sensitivity and efficiency in ESR analysis [54, 55]. This characteristic makes cyclic hydroxylamines suitable for detecting ROS in complex biological system, including pulmonary vasculature. For instance, using ESR/spin probe technique, Weissmann et al. have made the effort to detect reactive oxygen species, mainly superoxide, in isolated rabbit and mouse lungs [56]. In this study, the isolated and ventilated rabbit and mouse lungs were perfused with CPH as a spin probe, which was oxidized by ROS to form ESR-active $CP^{\bullet}$ radicals for the following ESR analysis. Using this method, the researchers demonstrated that chemical-induced pulmonary vasoconstriction is caused by superoxide generated from NADPH oxidases. In a more recent study, ESR analysis was also used to detect and quantify the superoxide levels in isolated mouse pulmonary arterial smooth muscle cells using another cyclic hydroxylamine spin probe 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH) [57]. Using this method, the authors demonstrated that the productionn of ROS is involved in pulmonary vascular remodeling during pulmonary hypertension. These studies suggested that perfusing isolated lungs from animal model with spin probes is a practical way for the detection of intravascular ROS release by ESR analysis.

In addition to spin probes, a spin-trapping technique was developed in the early 1970s, in which the primary free radicals are allowed to react with spin-trapping agents to form radical spin-trapping adducts prior to direct ESR detection [58–62]. The spin trapped adducts are newly formed radicals but are much more stable with longer lifetime that could thus be detected by ESR analysis. For example, POBN (α-[4-pyridyl 1-oxide]-*N*-tert-butyl nitrone) is a widely used spin trap agent that can react with various carbon-centered radicals to form POBN/radical adducts (Scheme 3) [63–71]. Sato et al. used ESR and POBN spin trapping to detect free radicals formed during lung injury caused by *Pseudomonas aeruginosa* and lipopolysaccharide in mouse and rat model [72, 73]; Arimoto et al. also employed ESR/spin trapping to detect radical production during diesel exhaust particles and lipopolysaccharide-induced lung injury [74]. In addition to POBN, there are also many other types of spin trap agents, mainly nitrone or nitroso compounds, including PBN (α-phenyl-tert-butylnitrone), MNP (2-methyl-2-nitrosopropane), TMPO (3,3,5,5-tetramethylpyrroline-N-oxide) and DMPO (5,5-dimethyl-1-pyrroline N-oxide) etc. They have also been widely used to detect free radicals generated during in physiological and pathological conditions [58–62, 72–76].

Although the ESR/spin trapping technique has become a common and powerful tool for the study of free radical species, it also suffers from some critical limitations. First of all, the ESR spectrum of spin trapping adducts lacks of specificity which makes unambiguous identification of primary free radicals impossible. This is because after adding spin traps, the ESR signals will be originated from the radical/spin trap adducts, instead of the primary free radicals. As long as the same spin trap is used, the ESR spectrum will be nearly identical independent of the type of primary free radicals. Secondly, the reaction rate between spin traps and free radicals are usually very low, and thus a high concentration of spin trap agents is required in order to generate ESR detectable signals, which limited the application of spin trap in biological study. In addition, the spin traps may undergo background reaction with various components in complex biological system, which interferes with the experiment outcome.

To overcome the limitation of nonspecificity of ESR-spin trap technique, Qian's group made a breakthrough in combining ESR/spin trapping



Scheme 3 Reaction between POBN (a nitrone spin trap reagent) with free radical species to form a spin trapping adduct for ESR analysis

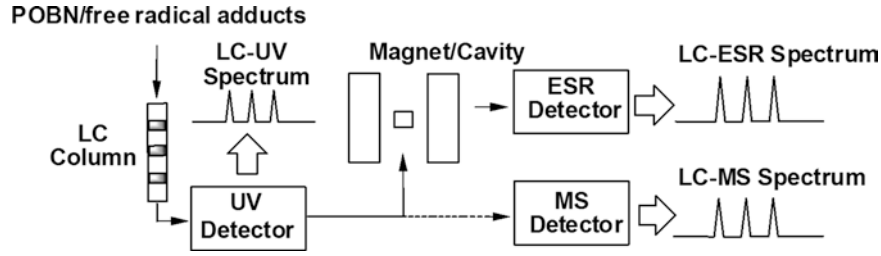
technique with HPLC and MS analysis in which individual free radical species are trapped with POBN, separated in HPLC column according to their distinct chromatographic behaviors, followed by ESR and MS detection to obtain detailed structural information from ESR spectrum and MS fragmentation (Scheme 4) [63–71]. Using the refined ESR-spin trapping/HPLC/MS combined technique, various free radical species produced from cyclooxygenase-catalyzed lipid peroxidation have been successfully detected and identified [70, 71, 77]. Furthermore, those lipid-derived radical by-products were recently found to play important roles in inhibiting cancer growth, including colon cancer and pancreatic cancer [77–80]. These studies suggested that the ESR-spin trapping/HPLC/MS combined technique has a huge potential to be employed for detecting free radical species in various disease models, including the pulmonary vasculature dysfunction.

In addition to ESR/spin trapping analysis, an immuno-spin trapping technique has also been developed in which protein or DNA-centered radicals are trapped by DMPO and allowed to react with anti-DMPO antibody followed by biological measurement such as western blots and immunofluorescence. This method does not require expensive ESR equipment, and provides unique specificity for the in situ and real time detection of protein or DNA-centered radicals in biological studies [81–86]. Recently, the immuno-spin trapping technique has been applied in the study of various disease models including ischemia–reperfusion injury, asthma, diabetes, obesity as well as endotoxin-induced experimental acute respiratory distress [87–93]. Therefore, the immuno-spin trapping technique shows a great potential for the study of redox signaling in physiological and pathological conditions.

3 Summary

Redox signaling and the production of ROS play important roles in regulating pulmonary vasculature constriction, structural remodeling, and cell proliferation during various physiological and pathological processes. The development and improvement of state-of-the-art techniques for ROS detection thus provide us with powerful tools for investigating the implications of redox signaling in pulmonary vasculature disorders. Thus, it is very important for us to understand the principles, individual advantages as well as limitations of various ROS detection methods in order to select proper methods for the study of specific ROS of our interest.

In this chapter, we review currently commonly used methods and techniques for the detection of ROS produced in vitro and in vivo, including chemiluminescence-based and fluorescence-based assay, cytochrome c reduction, genetically encoded fluorescent probes, and ESR/spin trapping technique. As demanded for the study in complex biological systems, many of these methods have been modified to more sensitive and target-specific versions, making them more powerful tools in the study of redox signaling in pathological conditions, including pulmonary vasculature dysfunction. In fact, many of these methods have been successfully applied in the study of redox signaling in pulmonary vasculature as we discussed above, which greatly advanced our knowledge in this field. However, more attention still need to be paid on those advanced techniques with great potential in ROS study, such as



Scheme 4 ESR-spin trapping, LC/ESR/MS combination technique for free radical detection

genetically encoded fluorescent probes and ESR/spin trapping technique. Continuous improvement of these techniques on their sensitivity and species/location-specificity is essential, as it will lead to a more comprehensive understanding on the diverse and complex functions of ROS as important signaling molecules in living system.

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